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TITLE OF INVENTION

PSEUDOTYPED RETROVIRAL VECTOR FOR GENE THERAPY OF CANCER

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☒ is attached hereto.
 - b. ☒ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☒ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☒ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). **UNEXECUTED**
10. ☒ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information: Copy of International Search Report; Fourteen (14) sheets of drawings included in the Published Application

EXPRESS MAIL CERTIFICATE NO.: EL 920250846 US DATE OF DEPOSIT: October 22, 2001

**PSEUDOTYPED RETROVIRAL VECTOR FOR
GENE THERAPY OF CANCER**

BACKGROUND OF THE INVENTION

5 (a) Field of the Invention

The invention relates to retroviral expression vectors and more particularly to pseudotyped retroviral vectors for gene therapy of cancer.

(b) Description of Prior Art

10 Tumor cells modified to express the Herpes Simplex Virus Thymidine Kinase gene (TK) acquire the ability to convert the non-toxic nucleobase analog gancyclovir (GCV) to its cytotoxic metabolite gancyclovir-phosphate. Cells genetically engineered to express this "suicide" gene are eliminated if exposed to gancyclovir. Experimental brain tumor implants consisting of a mixture of unmodified tumor cells with TK-expressing cells also regress following
15 gancyclovir treatment without harm to adjacent normal tissue. This phenomena, where a minority of TK-expressing cells lead to the death and elimination of adjacent tumor cells not expressing TK, has been termed the "bystander effect".

The "bystander" effect is dependent, in part, on cell-cell contact and on intercellular communications - gap junctions - through which gancyclovir-phosphate can circulate between TK-positive and TK-negative tumor cells.
20 Phagocytosis of gancyclovir-phosphate laden cell debris by adjacent tumor cells also leads to cell death. Blood vessel endothelial cells within or adjacent to the tumor may also acquire TK, and their destruction with gancyclovir therapy, thus, may also contribute to tumor regression. "Suicide" tumors release inflammatory
25 cytokines which promote hemorrhagic necrosis in local, but non-contiguous, tumor deposits. Furthermore, tumors undergoing a necrotic death, as opposed to apoptosis, will up-regulate the expression of proteins such as hsp70, IL10 and IL12, which may enhance immune recognition and rejection. Necrotic tumors may be infiltrated with a wide assortment of immunocompetent cells such as
30 CD4+ lymphocytes, CD8+ lymphocytes, NK cells and Antigen Presenting Cells. These infiltrating cells may take part in a tumor-specific immune response which is an important component of the local as well as distant anti-tumor immune

bystander effect (Moolten, F.L., *Cancer Research*, 46: 5276-5281, 1986). Intracerebral tumors are also susceptible to immune clearance following suicide gene expression, suggesting that the brain is not an immune sanctuary for cancer. Therefore, tumor-targeted suicide gene delivery leads to eradication of a defined tumor deposit if a sufficient number of targeted cells express the suicide gene. Malignant brain tumors are an appealing target for suicide gene delivery, since the entire malignancy is confined to the brain and amenable to eradication by the bystander effect. Key components for the success of this strategy are the genetic vector from which the suicide gene is expressed and its delivery vehicle.

Viral vectors remain the most efficient means to introduce genetic material in tumor cells *in vivo*. This is usually achieved by direct intra-tumoral or intravenous injection of a viral particle suspension. Among viral vector delivery platforms, adenoviruses are among the most studied for tumor-targeted gene delivery. Adenoviruses can be concentrated to high titers, which facilitates delivery of large viral doses to tumors. However, because of their ability to disseminate beyond local injection site and to transduce contiguous normal brain, including astrocytes, neurons and ependymal cells, suicide gene expression may lead to significant toxicity following gancyclovir treatment.

Recombinant retroviral vectors are well characterized as vehicles for tumor-targeted gene delivery. Retroviruses can integrate only in cells undergoing mitosis shortly after infection (Miller, D.G. et al., *Molecular & Cellular Biology*, 10: 4239-4242, 1990). Quiescent cells - such as normal brain tissue adjacent to a targeted tumor deposit - will be refractory to gene transfer and spared from subsequent toxicity (Culver, K.W. et al., *Science*, 256: 1550-1552, 1992). For this reason, retroviral vectors have been extensively used in human clinical trials studying suicide gene delivery to malignant brain tumors. Limitations to the use of retroviruses are: their inability to infect cells which do not express the retroviral receptor and, the low particle concentration in clinical-grade viral preparations. Clinical-grade retroviral particle preparations usually have titers $<10^7$ particles/ml. Assuming that a target tumor having a 1 cm diameter contains at least 10^8 cells, it would be necessary to inject intra-tumorally at least >10 ml of viral particle

preparation to deliver an equal number of viral particles. This logistical impediment to retrovirus delivery has been addressed by directly injecting murine retroviral producer cells (VPCs) into tumors *in vivo*, the idea being that locally produced viral particles could transduce cancer cells. Though this gene delivery approach led to cures in a rat model of brain cancer, this was probably achieved as a consequence of delivering as many VPCs as there were tumor cells (Culver, K.W. et al., *Science*, **256**: 1550-1552, 1992). In human clinical trials, where this strategy was duplicated by injecting amphotropic VPCs with a titer of 1×10^5 cfu/ml, low - albeit detectable - TK gene transfer efficiency was noted in tumor cells. Furthermore, a specific immune response against VPCs was elicited (Ram, Z. et al., *Nature Medicine*, **3**: 1354-1361, 1997). Although "suicide" retrovectors are "safe", implantation of VPCs as a means to deliver retroviruses is of limited efficacy. Poor suicide gene transfer to tumor cells is a major impediment to therapeutic utility.

15 Retroviruses which incorporate the Vesicular Stomatitis Virus G (VSVG) protein differ from traditional murine retroviral pseudotypes by their high affinity for a wide assortment of eukaryotic cells. This is primarily due to the ability of VSVG to recognize membrane phospholipid as a minimal receptor. Unlike standard murine retroviruses, VSVG retrovectors are also relatively resistant to deactivation by human complement (Ory, D.S. et al., *Proceedings of the National Academy of Sciences of the United States of America*, **93**: 11400-11406, 1996). Furthermore, like adenoviruses, VSVG-typed retroviruses can be concentrated to high titers by centrifugation and frozen/thawed without loss of activity. The VSVG pseudotype does not alter the retroviral genome's restricted targeting of cycling cells.

25 It would be highly desirable to be provided with a suitable delivery vehicle for suicide gene transfer, combining high titer, particle stability and tumor-specificity.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a suitable retroviral vector for gene therapy of a cancer.

For example, VSVG-typed retroparticles may be suitable for
5 delivering a therapeutic gene to a tumor tissue.

For example, the cancer may be a brain cancer.

Examples of therapeutic genes include suicide genes.

For example, an HSVTK-expressing retrovector and VSVG-
pseudotyped retroparticles were constructed. Human glioma cell lines can be
10 transduced *in vitro* and express functionally significant amounts of HSV TK.
Concentrated retroparticles were administered intra-tumorally in a rat model of
brain cancer and a significant survival benefit was noted following gancyclovir
therapy.

The retrovector may incorporate the AP2 expression vector. The AP2
15 expression vector allows for a high level expression of a transgene and
incorporates a reporter gene for monitoring of the transgene expression *in vitro*
and *in vivo*. Furthermore, the reporter protein allows for a sorting of producer
cells and facilitates the measurement of the retroviral titer.

In accordance with the present invention, retrovectors which may be
20 used include, without limitation, AP2 expression vector, AP2 derivatives thereof
such as its first derivative AP3 which includes the HSVRK suicide gene. Other
derivatives include MD1 which is a AP2 derivative which incorporates human
GMCSF, JGH2 which expresses a novel GFP-HSVTK fusion protein, JGH2
derivatives thereof which incorporate immunomodulatory genes as well as the
25 GFP/TK fusion protein. AP2 derivatives incorporate genes of therapeutic interest
for the treatment of cancer.

Preferred AP2 expression vector derivatives include, without
limitation, the following:

HSV thymidine kinase (AP3);
30 GMCSF (MD1);
RARB2;
IRF3;
IRF3-5d;
MCP1;
35 Rantes;

MIP1alpha;

MIP1beta;

MCP1.

Preferred JGH2 expression vector derivatives include, without
5 limitation, the following:

GMCSF (MD2);

IRF3 (AP6);

IRF3/5D (AP7).

10 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figs. 1A-1C illustrate schematic representations of plasmids and retrovectors. Fig. 1A: AP2 plasmid retrovector serves as a template for the co-expression of the EGFP reporter and of a linked cDNA in eukaryotic cells. The cDNA of interest is inserted in the multiple cloning site upstream of the IRES.
15 Fig. 1B: pTKiGFP is a derivative of AP2 which contains the HSVTK gene. Transfection of this plasmid into retroviral packaging cells will lead to the production of replication-defective retroparticles. Fig. 1C: Target cells transduced with vTKiGFP will integrate the retrovector in their genomic DNA. The DNA structure (flanked by LTRs) and coding sequences are depicted.

20 Fig. 2 illustrates flow cytometric analysis of vTKiGFP transduced glioma cells. UWR7 human glioma cells were transduced with vTKiGFP and subsequently analyzed by flow cytometry for green fluorescence, as described in "Materials and Methods". GFP serves as a reporter of retrovector expression in transduced cells.

25 Fig. 3 illustrates Southern Blot analysis on vTKiGFP transduced glioma cells. Following transduction with vTKiGFP, the retrovector will integrate into genomic DNA. Digest of genomic DNA with NheI, which cuts once in each flanking LTR, and subsequent probing of Southern blot with a vector complementary sequence will allow detection of integrated proviral sequences
30 with a predicted size of 4kb (schematic at right). Left, Southern blot analysis of transduced (+) and untransduced (-) UWR7 cells with a GFP cDNA-specific probe, as described in "Materials and Method". Molecular weights are indicated.

Fig. 4 illustrates growth suppression of human glioma cells with gancyclovir. The indicated human glioma cell lines were transduced with vTKiGFP (open squares) or the control retrovector vDHFRiGFP (open circle). These and untransduced controls (open diamonds) were subsequently exposed to gancyclovir for 6 days, and cell survival was measured by the MTT assay as described in "Materials and Methods". Percent survival is plotted against gancyclovir concentration (log scale). Data points, mean survival measured in three separate experiments; bars, SD. SD smaller than data point icon are not displayed.

Fig. 5 illustrates flow cytometric analysis of 293AP3 producer cells. 293GPG packaging cells were stably transfected with pTKiGFP and a Zeocin resistance plasmid. A mixed population of Zeocin resistant 293AP3 cells was generated and characterized for GFP expression by flow cytometry as described in "Materials and Methods". Percent GFP+ cells is indicated. These cells were subsequently utilized to generate vTKiGFP stock for concentration and *in vivo* delivery.

Fig. 6 illustrates transduction of glioma cells with concentrated vTKiGFP retrovector stocks. vTKiGFP retroparticles were collected and concentrated to 84 and 1000 fold (volume/volume) as described in "Materials and Methods". 1X and 84X virus stock were diluted (as indicated on left) in a final volume of 1 ml and applied to 2.3×10^5 UWR7 cells in a 24 well dish. Three days following a single application of vector, cells were analyzed for GFP expression by flow cytometry. Percent GFP+ is indicated in histogram figures. Dilutions of 1000X stock was applied to 5.4×10^5 C6 glioma cells and analyzed three days later for GFP expression. Titer extrapolated from these experiments were: 1X: 2.9×10^7 cfu/ml, 84X: 2.2×10^9 cfu/ml, 1000X: 2.3×10^{10} cfu/ml.

Fig. 7 illustrates *in vivo* transduction of C6/lacZ tumors with vTKiGFP. Brain tumors were harvested post-mortem as described in Materials and Methods. TOP (panel A, B), tumor from a control rat which received vTKiGFP without subsequent treatment with GCV (rat was sacrificed on day 30 post tumor implantation due to morbid state). MIDDLE (Panel C, D), tumor from

a control rat which did not receive vTKiGFP but was treated with GCV (rat was sacrificed on day 43). BOTTOM (Panel E, F), tumor from a test rat which received vTKiGFP and subsequent treatment with GCV which suffered symptomatic recurrent tumor (rat was sacrificed on day 82). GFP expression (panels A, C, E) was compared to subsequent histochemical staining of C6/lacZ tumor cells with the substrate X-gal (panels B, D, F). Magnification of 100X for all photomicrographs.

Fig. 8 illustrates Kaplan-Meier survival curve of rats with experimental glioma. Sprague-Dawley rats received 2×10^4 C6/lacZ glioma cells by stereotactic injection in the right brain hemisphere as described in "Materials and Methods". Six days later, eighteen animals were administered 9 μ L of 1000x vTKiGFP stock in the same stereotactic coordinates as the previous C6/lacZ implant. 48 hours later, test animals (n=12) received GCV 50mg/kg twice daily for 5 days followed by 50mg/kg once daily for 5 more days. The other animals (n=6) were administered saline only. In a separate experiment, a supplementary cohort (n=5) received a C6/lacZ glioma implant followed 9 days later by GCV treatment (no retrovector administered). The survival seen in the test group (vTKiGFP + GCV) is significantly greater than that in either control groups ($p < 0.001$ by Log rank). There is no significant difference in survival between the two control groups.

Fig. 9 illustrates retrovectors. Panel A. nucleotide sequence of DNA linker region spanning the 3'-end of GFP and start codon of HSVTK. Nucleotides derived from GFP cDNA are in bold and underlined with their translation product also in bold. The sequence point of fusion between the 3' end of GFP and the 5' untranslated region of HSVTK cDNA sequence is depicted. Predicted 24 aminoacid linker is depicted. HSV TK start codon and coding sequence are identified in bold text and HSVTK caption. Panel B. Schematic representation of plasmid constructs. Left, pGFP retrovector encodes for GFP only; Center, pTKiGFP is a bicistronic expression vector incorporating HSVTK and GFP. Right, pGFPTKfus incorporates the coding sequences for a GFP and HSVTK fusion protein. All three plasmid constructs were utilized to generate

stable retroviral producers with the 293GPG packaging cell line as described in Materials and Methods.

Fig. 10 illustrates Southern blot analysis of vGFPTKfus transduced cells. After transduction with vGFPTKfus, the retrovector will integrate into genomic DNA. Below, Digest of genomic DNA with NheI, which cuts once in each LTR, and subsequent probing of Southern blot with a vector complementary sequence will allow detection of integrated proviral sequences with a predicted size of 3.7 kb. Top, Southern blot analysis of transduced (+) and untransduced (-) human A549 cells with a GFP cDNA-specific probe. Arrow indicates band of predicted size. Molecular weights are indicated on left.

Fig. 11 illustrates Flow cytometry of retrovirally-transduced DA3 cells. DA3 mouse mammary carcinoma cells were transduced with either vTKiGFP, vGFPTKfus or vGFP in a manner which leads to 100% gene transfer efficiency. Stably transduced polyclonal cell populations were subsequently analyzed by flow cytometry for green fluorescence as described in Materials and Methods. GFP serves as a reporter of retrovector expression in transduced cells and the Mean Fluorescence Intensity (MFI) of the analyzed populations is indicated in the top right of each panel.

Fig. 11 illustrates fluorescent microscopy of vGFPTKfus engineered cells.

Fig. 12 illustrates Western blot analysis. The same transduced DA3 cells analyzed by flow cytometry (Fig. 3) were utilized for Western blot analysis of HSVTK protein expression. Equal amounts of total protein obtained from whole cell lysates were separated by gel electrophoresis and immunoblotted with an anti-HSVTK polyclonal antisera as detailed in Materials and Methods. Molecular weight markers (kd) are depicted on the left.

Fig. 13 illustrates gancyclovir growth suppression assay. The vTKiGFP (filled square), vGFPTKfus (filled triangle) and vGFP (filled circle) transduced DA3 mouse mammary carcinoma cells were exposed to the prodrug gancyclovir for 6 days and cell survival was measured with the MTT assay as described in Materials and Methods. Percent survival is plotted against GCV

concentration (log scale). DA3 transduced with a GFP only vector serve as negative controls. Data points, average +/- SD of three experiments is depicted, error bars smaller than icons are not shown.

5 DETAILED DESCRIPTION OF THE INVENTION

Direct *in vivo* tumor-targeting with "suicide" viral vectors is limited by either inefficient gene transfer [i.e. retroviral vectors] or indiscriminate transfer of a conditionally toxic gene to surrounding non-malignant tissue [i.e. adenoviral vectors]. Retrovectors pseudotyped with the Vesicular Stomatitis Virus G protein (VSVG) may serve as a remedy to this conundrum. These retroviral particles differ from standard murine retroviruses by their very broad tropism and the capacity to be concentrated by ultracentrifugation without loss of activity. A VSVG-typed retrovector can be utilized for efficient and tumor specific Herpes Simplex Virus Thymidine Kinase (TK) gene delivery *in vivo*. A bicistronic retroviral vector which expresses TK and Green Fluorescence Protein (pTKiGFP) was constructed. The 293GPG packaging cell line was utilized to generate vTKiGFP retroparticles. In cytotoxicity assays, vTKiGFP-transduced human glioma cell lines were sensitized to the cytotoxic effects of gancyclovir (GCV) 10,000 fold. Subsequently, the virus was concentrated by ultracentrifugation to a titer of 2.3×10^{10} cfu/ml. The anti-tumor activity of vTKiGFP retroparticles was tested in a rat C6 glioma model of brain cancer. Concentrated retrovector stock (9 μ L volume) was injected stereotactically in pre-established intra-cerebral tumor. Subsequently, rats were treated with GCV for 10 days. Control rats (no GCV) had a mean survival of 38 days (range 20-52 days). Sections performed on post-mortem brain tissue revealed large tumors with evidence of high efficiency retrovector transfer and expression (as assessed by GFP fluorescence). Fluorescence was restricted to malignant tissue. In the experimental group (GCV treated), 8/12 remain alive and well >120 days post glioma implantation. The vTKiGFP is very efficient at transducing human glioma cell lines *in vitro* and leads to significant GCV sensitization. Recombinant retroviral particles can be concentrated to titers which allow *in vivo* intra-tumoral delivery of large viral

doses. The therapeutic efficiency of this reagent has been demonstrated in a pre-clinical model of brain cancer.

MATERIALS AND METHODS

Cell lines and plasmids

5 pCMMP-LZ plasmid (Jeng-Shin Lee and Richard C. Mulligan, unpublished), pJ6 Ω bleo plasmid and 293GPG retroviral packaging cell line were generous gifts from Richard C. Mulligan (Children's Hospital, Boston, MA). MSCV-Neo plasmid (Hawley, R.G. et al., *Gene Therapy*, **1**: 136-138, 1994) and BSICZSVPA plasmid (Ghattas, I.R. et al., *Molecular & Cellular Biology*, **11**:
10 5848-5859, 1991) were kindly provided by Robert G. Hawley (The Toronto Hospital, Toronto, ON). SKI-1, SKMG-4, SKMG-1, T98G, UW28 & UWR7 human glioma cell lines were generously provided by Lawrence Panasci (Lady Davis Institute for Medical Research, Montreal, QC). C6 & C6/lacZ glioma cells originate from ATCC. pMC1TK plasmid was graciously provided by Gerald
15 Batist (Lady Davis Institute for Medical Research, Montreal, QC). HaL22Y plasmid was kindly provided by Raymond L. Blakley (St. Jude Children's Research Hospital, Memphis, TN).

Retrovector design and synthesis

A plasmid encoding for a bicistronic, non-splicing murine retrovector
20 which incorporates a multiple cloning site - allowing insertion of cDNA of interest - linked to the Enhanced Green Fluorescence Reporter (AP2) was engineered. The synthesis of AP2 is as follows. The 805 bp EGFP cDNA was excised by Eco47-3 and NotI digest of pEGFP-N1 (Clontech, Palo Alto, CA) and ligated into the MSCV (Hawley, R.G. et al., *Gene Therapy*, **1**: 136-138, 1994)
25 retroviral plasmid to generate MSCV-EGFP. The 555 bp Internal Ribosomal Entry Site (IRES) was excised from the BSICZSVPA plasmid (Ghattas, I.R. et al., *Molecular & Cellular Biology*, **11**: 5848-5859, 1991) by SacII-NcoI digest and cloned in to SacII-NcoI cut MSCV-EGFP to generate MSCV-IRES/EGFP. MSCV-IRES/EGFP was digested with SpeI-AscI to generate a 2524 bp fragment
30 encompassing part of the 5' untranslated region of the retrovector, the IRES, EGFP and most of the 3' LTR. This insert was ligated with a 4169 bp fragment

from SpeI-AscI cut pCMMP-LZ - an unpublished MFG-based retrovector - to generate AP2 (Fig. 1A). AP2 is designed to co-express an inserted cDNA with the EGFP reporter within a bicistronic framework. The EGFP serves as a reporter of provirus transfer and expression in target cells. The viral vector generated is non-splicing. The pMC1TK plasmid was cut with BglII-BsaW1 to generate a 1207 bp fragment containing the HSVTK cDNA (excluding polyadenylation signal) and was ligated into BglII-XmaI-cut AP2 to generate pTKiGFP (Fig. 1B). The retroviral genome produced from pTKiGFP will not incorporate the CMV promoter element. Transduction of target cells with pTKiGFP-derived retroviral particles (vTKiGFP) will lead to the stable incorporation of LTR flanked proviral genome (Fig. 1C). The pMSCV-DHFR (L22Y)/IRES/EGFP vector (pMSCV-DIG) was derived by incorporating the 654 bp BamHI-XhoI DHFR (L22Y) cDNA from Ha-L22Y into BglII-SalI cut MSCV-IRES/EGFP.

Production of VSVG-pseudotyped retroviral particles and concentration

Recombinant VSVG-pseudotyped retroparticles were generated either by transient or stable transfection of the 293GPG packaging cell line (Ory, D.S. et al., *Proceedings of the National Academy of Sciences of the United States of America*, **93**: 11400-11406, 1996). 293GPG cells are maintained in 293GPG media [DMEM (Gibco-BRL, Gaithersburg, MD), 10% heat-inactivated FBS (Gibco-BRL) supplemented with 0,3 mg/ml G418 (Mediatech, Herndon, VA) and 2 µg/ml puromycin (Sigma, Oakville, ONT), 1 µg/ml tetracycline (Fisher Scientific, Nepean, ONT) and 50 units/ml of Pen-Strep]. For transient production of retroparticles, 293GPG cells were transfected with 5 µg plasmid retrovectors with the use of lipofectamine (Gibco-BRL). Transient transfections were done in tetracycline-free media and viral supernatant collected daily for 1 week, 3 days following transfection. Stable producer cells were generated by co-transfection of 4µg FspI linearized retrovector plasmid and 1:25 ratio of pJ6ΩBleo plasmid. Transfected cells were subsequently selected in 293GPG media supplemented with 100 µg/ml Zeocin (Invitrogen, San Diego, CA) as described (Ory, D.S. et al., *Proceedings of the National Academy of Sciences of the United States of America*, **93**: 11400-11406, 1996). Resulting stable polyclonal producer populations were

utilized to generate high titer virus. All viral supernatants were filtered with 0.45 micron syringe mounted filters (Gelman Sciences, Ann Arbor, MI) and stored at -20°C . Concentration of VSVG retroparticles was performed as previously described (Ory, D.S. et al., *Proceedings of the National Academy of Sciences of the United States of America*, **93**: 11400-11406, 1996). In brief, previously harvested supernatant was thawed and 10 ml aliquots were centrifuged at 25,000 rpm in a SW41T1 rotor (Beckman Instruments Inc.) at 4°C for 90 minutes. Viral pellets were resuspended overnight in 100 μL serum-free RPMI (Gibco-BRL) at 4°C , pooled and concentrated through a second centrifugation. Concentrated virus was aliquoted and stored at -80°C . Viral preparations were devoid of RCR by EGFP marker rescue assay utilizing supernatant from transduced UWR7 cells. Transduction of glioma cells, flow cytometry and southern blot analysis

Human glioma cell lines were plated at 2×10^4 cells per well in a 24 well dish and allowed to adhere. Media was removed and replaced with 500 μL of thawed, retrovirus conditioned media collected from transiently transfected 293GPG. Polybrene (Sigma) was added to a final concentration of 6 $\mu\text{g}/\text{ml}$. This procedure was repeated daily for three consecutive days. Stably transduced cells were subsequently expanded. No clonal selection was performed, and mixed populations of transduced cells were used for all subsequent experiments. Flow cytometric analysis was performed within two weeks following transduction to ascertain retrovector expression and gene transfer efficiency as measured by GFP fluorescence. In brief, adherent transduced cells were trypsinized and resuspended in RPMI at $\sim 10^5$ cells per ml. Analysis was performed on a Epics XL/MCL Coulter analyzer. Live cells were gated based on FSC/SSC profile and analyzed for GFP fluorescence. Southern blot analysis was performed on 15 g of overnight NheI digested genomic DNA extracted from stably transduced cells as well as untransduced control cells. Blots were hybridized with a P^{32} labeled, full-length 700 bp GFP cDNA probe, washed and exposed on photographic film.

Growth suppression assays

Stably transduced test and control cells were trypsinized and plated at a density of 1000 cells per well in a flat bottomed tissue-culture treated 96 well

plate (Costar corporation, Cambridge, MA). Clinical-grade gancyclovir (GCV, Hoffman-Laroche, Mississauga, ONT) was added to achieve a range of concentrations from 0.01 to 5000 $\mu\text{g/ml}$ in a final volume of 100 μL of RPMI/10% FBS. Cells were incubated at 37°C and media was replaced with fresh GCV after three days for a total exposure of 6 days. The percentage of surviving cells was measured using a method based on the metabolism, by living cells, of the mitochondrial substrate 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) into formazan, which is detected by measurement of the optical density at 570nm. Percent Survival is calculated as follows [OD570 test - OD570 empty well]/[OD570 untreated cells - OD570 empty well] x 100. All data points were measured in triplicate in at least three separate experiments.

Titration of retrovector

Target glioma cells were plated at 2×10^5 cells per well in a 6 well tissue culture dish. The next day, cells from a test well were trypsinized and enumerated to determine baseline cell count at moment of virus exposure. Virus was serially diluted (range 100 to 0.001 μL) in a final volume of 1 ml of RPMI/10% FBS supplemented with 6 $\mu\text{g/ml}$ polybrene (Sigma) and applied to adherent cells. Flow cytometric analysis was performed 3 days later to determine the percentage of GFP+ cells. Viral titer (cfu/ml) was extrapolated from the test point in which non-saturating transduction conditions prevailed (i.e. transduction efficiency <80%). Titer (cfu/ml) was calculated as [(% GFP+ cells) X (cell number at initial viral exposure) / (viral volume in ml applied)].

Animal model of brain cancer, *In vivo* retrovector delivery and gancyclovir treatment

C6/lacZ glioma cells reproducibly generate lethal intra-cerebral tumors when injected in Sprague-Dawley rats. The constitutive β -galactosidase expression facilitates delineation (by X-gal staining) of tumor cells and extent of the tumor infiltrate in post-mortem brain sections. Adult Sprague Dawley rats were anesthetized with intraperitoneal injection of ketamine (50 mg/kg) and xylazine (2 mg/kg). C6/lacZ rat glioma cells (2×10^4 cells in 5 μL of HBSS) were injected intracranially into the frontal lobe using a Hamilton syringe in a stereo-

tactic apparatus (Kopf) over a period of 15 minutes. The coordinates used were 3.5 mm lateral to the bregma, 1.0 mm posterior to the coronal plane and 4.5 mm in depth of the dural surface. Six days post glioma cell implantation, rats were anesthetized and vTKiGFP (concentrated stock of 2.3×10^{10} cfu/ml) was injected of
5 into six different sites (1 mm apart) in the pre-established tumor guided by the previous stereotactic coordinates. A total volume of 9 μ l was injected in each tumor (6 x 1.5 μ l increment) and needle was left in place for at least 5 mins per increment (for a total of 30 mins per tumor). Two days after retrovector delivery, rats are treated with GCV 50 mg/kg intraperitoneally twice daily for 5 days fol-
10 lowed by 50 mg/kg once daily for another 5 days. After euthanasia, brains were removed and quickly frozen in isopentane chilled with liquid nitrogen. Coronal sections (10 μ m) were prepared. GFP activity was observed by epi-fluorescence microscopy and recorded photographically. Subsequently, sections were stained histochemically for β -galactosidase activity as previously described before
15 counter staining with hematoxylin and eosin.

RESULTS

Retrovector design and synthesis

The AP2 expression vector (Fig. 1A) allows the incorporation of a cDNA sequence in a Multiple cloning site (MCS) upstream of an Internal Ribo-
20 somal Entry Site (IRES) and the Enhanced Green Fluorescent Protein (EGFP) cDNA. The transcription initiation from a CMV promoter will lead to the production of a bicistronic mRNA incorporating both the inserted cDNA and the EGFP coding sequence. Translation of both coding sequences will be achieved from a single mRNA molecule, thereby ensuring co-dominant expression of both
25 protein products. Live cells expressing EGFP, which is detectable by fluorescence microscopy or flow cytometry, will co-express the linked gene product. Gene-modified cells can be implanted or transplanted in animal models and their localization and function be traced based on the expression of the EGFP protein. The AP2 expression vector incorporates a replication-defective retroviral packaging sequence and a retroviral 3' long terminal repeat (LTR). Transfection
30 of an appropriate retroviral packaging cell line can lead to production of

recombinant retroviral particles. Retroparticles can be generated either by transient transfection of packaging cell lines or alternatively, stable producer cell lines can be generated by co-transfection with a drug resistance plasmid. We have generated retroparticles by both methods with good success utilizing the 293GPG retroviral packaging cell line.

Retrovector transfer and expression in human glioma cell lines

The 293GPG packaging cell line was transiently transfected with pTKiGFP (Fig. 1B) and supernatant containing VSVG-typed retroparticles (vTKiGFP) was subsequently collected, filtered and frozen for storage. Human glioma cell lines (SKI-1, SKMG-4, SKMG-1, T98G, UW28 & UWR7) were transduced with three consecutive daily applications of thawed vTKiGFP supernatant. Six days post-transduction, polyclonal cell lines were subjected to flow cytometric analysis to determine the proportion of cells which expressed the GFP reporter protein. All polyclonal cell lines were 100% GFP-positive by FACS analysis, and transduced UWR7 cells serve as a representative example (Fig. 2). We have also found that GFP expression could be easily detected in live cultured cells by direct visualization with a tissue culture microscope fitted with an epifluorescence light source. Southern blot analysis confirmed that unarranged vTKiGFP vector integrated in chromosomal DNA of transduced target cells (Fig. 3). vTKiGFP transduced cells have been passaged in excess of 30 times without loss of GFP expression.

vTKiGFP Expression and Gancyclovir sensitization

HSV TK expression will lead to the conversion of the prodrug gancyclovir to its cytotoxic metabolite gancyclovir monophosphate. Cells which do not express this enzyme are refractory to gancyclovir toxicity. We compared the gancyclovir sensitivity of vTKiGFP transduced cells with unmodified parental cells as well as cells modified with a control, GFP-containing retrovector (vMSCV-DIG). Cells were plated in 96 well dish and exposed to gancyclovir for a period of 6 days. Live cell content was assessed colorimetrically by MTT assay and cell survival was expressed as a percentage relative to untreated cells. We have found that all vTKiGFP-expressing cell lines were sensitized to gancyclovir. Comparing

the GCV concentration which inhibits cell growth by 50% (IC50), we found that vTKiGFP transduced cells (all 6 cell lines aggregated) were up to 10,000 fold more sensitive to GCV than controls (IC50 tests: 0.004 ug/ml vs IC50 controls: 40 ug/ml, $p < 0.001$ by student t test) (Fig. 4). Growth rate for transduced and parental cell lines in the absence of gancyclovir were identical.

Concentration of vTKiGFP retroparticles

The most direct means of transducing a tumor *in vivo* is to inject the therapeutic retrovector intra-tumorally. If the aim is to transduce as many tumor cells as possible, it would be desirable to inject a concentrated vector stock to achieve a high local MOI. We determined if viable vTKiGFP retroparticles could be concentrated by ultracentrifugation as previously described. As a first step we transfected 293GPG cells with pTKiGFP and a zeocin resistance plasmid (pJ6bleo). A stably transfected, Zeocin-resistant polyclonal producer cell population (293AP3) was generated. Flow cytometric analysis for GFP fluorescence revealed that 42% of this mixed population stably expressed the pTKiGFP vector DNA (Fig. 5). Tetracycline withdrawal from the culture media will lead to the production of VSVG-typed vTKiGFP retroparticles. We collected retroparticle-containing media daily from the 293AP3 producer cells from days 3 to 8 following tetracycline withdrawal. Supernatant was cleared of cellular debris with a 0.45 μ filter and frozen. We have noted that twice daily media collection - as opposed to once daily - doubled the yield of retroparticles from producer cells following tetracycline withdrawal. Media were thawed, pooled and subjected to ultracentrifugation as described in Materials and Methods. Supernatant was concentrated 84 fold (20 mls to 0.24 ml) by ultracentrifugation. The concentration step raised titer from 2.9×10^7 cfu/ml to 220×10^7 cfu/ml as measured on UWR7 human glioma cells (Fig. 6). 84X concentrates were pooled and subjected to a second ultracentrifugation to achieve a final 1000X (100 ml initial volume to 0.1 ml final volume) concentration. Titer of 1000X retrovector was 2.3×10^{10} as determined on rat C6 glioma cells (Fig. 6). Concentrated retrovector aliquots were stored at -80°C until further use. We have observed that unmanipulated (unconcentrated) supernatant from tetracycline-deprived 293GPG

producer cells can be toxic to target cells if applied repeatedly. However, no toxicity was observed on target cells if concentrated supernatant was used for transduction purposes, even at the highest tested MOI (>100).

5 **Retrovector expression following intra-tumoral injection of concentrated vTKiGFP retroparticles**

Implantation of C6/lacZ glioma cells will reliably lead to the establishment of intra-cerebral tumors in immunocompetent Sprague-Dawley rats. This cell line will generate large local tumors which are uniformly lethal within 60 days following the initial stereotactic injection of 2×10^4 cells. Furthermore, 10 C6/lacZ cells constitutively express β -galactosidase which permits the assessment of tumor extent and local invasion in X-gal stained post-mortem brain sections. 18 rats received 2×10^4 C6/lacZ cells via stereotactic injection in the right brain hemisphere. Six days later, 9 μ L of 1000X vTKiGFP retrovector (2×10^{10} cfu/ml) was injected at the tumor site using the same stereotactic coordinates. Of these 15 rats, 6 were randomly chosen and treated with saline. Saline-treated control rats had an average survival of 38 days (range 20 to 52 days). Post-mortem examination of brain revealed macroscopic intra-cerebral tumors, except for 1 rat which died with leptomeningeal tumor spread 8 days after tumor injection (which was excluded from further analysis). Examination of fresh frozen brain sections 20 by epifluorescence microscopy shows that in all animals, a predominant proportion of glioma cells fluoresce green (Fig 7A), including distant micrometastasis. Normal surrounding brain tissue is bereft of green fluorescence. No green fluorescence was observed in untransduced brain tumors (Fig 7C).

Gancyclovir treatment of rats with vTKiGFP-targeted gliomas

25 Of 18 rats having received intra-tumoral vTKiGFP retrovector, 12 were subsequently treated with gancyclovir. Two days following retrovector injection, rats received gancyclovir 50 mg/kg intra-peritoneally twice daily for 5 days followed by 50 mg/kg once daily for another 5 days. Significant gancyclovir toxicity including transient limb paresis and otorrhagia, was noted in some rats in 30 the week following GCV treatment. Of 12 gancyclovir treated rats, two died within 10 days following drug treatment presumably from direct GCV toxicity

(both animals had brain tumors <1mm in diameter on post-mortem). The other 10 rats fully recovered from GCV toxicity. Two rats developed tumor relapses at the initial injection site and died of progressive disease at day 82. Examination of brain tissue sections on these late relapses, revealed focal GFP expression in the tumors (Fig 7E). Significantly enhanced survival was obtained as eight of 12 GCV-treated test rats (66%) remain long term survivors (>120 days). A supplementary control cohort of 6 rats implanted with C6/lacZ, but without subsequent retrovector administration, was treated with the same GCV regimen. These controls had an average life span of 47 days (range 31 to 63 days) (Fig. 8). With our experimental C6 glioma model, we have not observed a significant difference in average survival between the two control groups (saline controls vs GCV-treated null tumors, $p=0.37$ (Student t test)) suggesting that GCV treatment, on its own, does not have a measurable impact on survival, as has been suggested by others using 9L glioma implants. These differences may be due different biological properties of these two experimental glioma models.

DISCUSSION

Engineering tumor cells to express the Herpes Simplex Virus Thymidine Kinase cDNA will lead to their destruction if they are subsequently exposed to non-toxic nucleobase analogs such as gancyclovir. This "suicide" effect is accompanied by "bystander" toxicity on adjacent tumor cells not expressing TK, so that a minority of engineered tumor cells - perhaps no more than 10 to 25% - will lead to 100% tumor eradication. Clinical application of this therapeutic strategy requires relatively high efficiency TK gene transfer to pre-established tumors. Furthermore, "collateral" gene transfer to normal adjacent normal tissue would have to be curtailed to prevent GCV toxicity to normal brain tissue.

The affinity of recombinant retroparticles for target tissue is defined by the env protein. Murine amphotropic retroviruses, from which are derived many of the therapeutic retrovectors in glioma targeted gene delivery, will only bind target cells which express a specific inorganic phosphate transporter. If a target tumor does not express the retrovirus receptor, gene transfer - and therapeutic benefit - is unlikely to occur. Retroparticles which are pseudotyped with the

VSVG protein will adopt the wide host range of the vesicular stomatitis virus. The putative VSVG receptor on target cells - which is believed to be membrane phospholipid - is ubiquitously found in all eukaryotic cells. This has led the use of VSVG-pseudotyped retrovectors as gene delivery vehicles in wide assortment of mammalian, non-mammalian and invertebrate cells (Yee, J.K. et al., Proceedings of the National Academy of Sciences of the United States of America, 91: 9564-9568, 1994). A major advance in pseudotyping retrovectors with VSVG was achieved when a practical "transient" VSVG retroviral packaging cell line was designed. The subsequent publication of a reliable "stable" high-titer VSVG packaging cell lines - including 293GPG (Ory, D.S. et al., *Proceedings of the National Academy of Sciences of the United States of America*, 93: 11400-11406, 1996) - has allowed the development and characterization of pseudotyped retrovectors for a wide variety of gene transfer applications (Hopkins, N., *Proceedings of the National Academy of Sciences of the United States of America*, 90: 8759-8760, 1993), including tumor cell-targeted gene delivery.

We have examined the utility of a VSVG-pseudotyped suicide retrovector for glioma-targeted gene delivery. To facilitate analysis of vector transfer efficiency and expression in target cells, we engineered a retroviral expression vector which incorporates HSVTK and the EGFP reporter cDNA within a bicistronic transcript (pTKiGFP). We have found that co-dominant expression of the HSVTK cDNA and of the EGFP reporter facilitates a wide assortment of procedures associated with synthesis and characterization of viral vectors. Among these, are the ability to measure endpoint titer from stable retroviral producer cells (Fig. 6) as well as potential use for selecting GFP+ producer cells with a cell sorter device. We have also found that the EGFP reporter can serve as a sensitive marker of retrovector expression in targeted tissue *in vitro* (Fig. 2) as well as *in vivo* (Fig. 7).

We generated a stable retroviral vTKiGFP producer cell line (293AP3) derived from the 293GPG packaging cell line (Fig. 5). Upon tetracycline withdrawal, this retroviral producer cell line will express the VSVG envelope protein and generate pseudotyped retroviral particles. We found that VSVG-pseudotyped

retroparticles incorporating vTKiGFP will lead to high efficiency retrovector transfer to human glioma cell lines *in vitro*. In contrast with standard transfection techniques, or with the use of more "standard" retroviral pseudotypes, we have not required dominant selection of subpopulations of cells to achieve 100% transgene-positive cell populations. Retroparticle conditioned media collected from 293GPG cells transiently transfected with pTKiGFP, was used to generate vTKiGFP transduced glioma cell lines. We noted that transducing glioma cells with concentrated retrovector with a single application at a MOI of ~5 led to more than 90% gene transfer in targeted cells (Fig. 6). Gene expression was durable as assessed by persistent GFP expression (>30 passages) and by functional HSVTK expression, rendering VSVG-associated pseudotransduction unlikely. Having generated vTKiGFP transduced cell lines, we confirmed that the proviral genome integrated unrearranged by southern analysis, demonstrating the stability of the viral vector as designed (Fig. 3). This of some importance especially in light of recent reports documenting rearranged "suicide" retroviral vectors as a cause of gancyclovir resistance in transduced tumors. Virtually all glioma cell lines transduced with vTKiGFP acquired substantial and significant sensitivity to gancyclovir *in vitro* (Fig. 4). Our experimental design based on the use of polyclonal transduced cell populations for cytotoxicity assays, supports the hypothesis that vTKiGFP gene transfer, on the average, will express biologically significant levels of TK in a gene-modified cell. Neither the transduction process (with a control retrovector), nor expression of the GFP reporter, on their own, sensitizes cells to gancyclovir (Fig. 4).

Important characteristics of VSVG pseudotyped retroparticles are their ability to sustain concentration by ultracentrifugation and repeated freeze/thaw without loss of activity. These properties have allowed us to collect retroparticle conditioned media on a daily basis following tetracycline withdrawal from the 293AP3 producer cell line. Retroparticle-containing media was frozen and stored until further use. Large volumes of frozen supernatant can be thawed, pooled and subjected to at least two cycles of centrifugation with efficient retrovector recovery. We concentrated 100 mls of media to a final volume of 0.1 ml (1000X

concentration on volume basis). This was accompanied by a 800 fold increase in titer from 2.9 to 2300×10^7 cfu/ml. We noted that supernatant from tetracycline-deprived 293AP3 producer cells could be toxic to target cells if applied repeatedly. We also observed this phenomena with other 293GPG-derived producer cells. Interestingly, we observed that concentrated retroparticles, which had been resuspended in serum-free media did not have this property although they would be delivered at a MOI higher than that achievable with the unconcentrated supernatant. This suggests that supernatant from tetracycline-deprived 293GPG cells contains toxic constituent(s) which are readily discarded upon concentration procedure.

To test the therapeutic usefulness of this reagent, we utilized a rodent model of brain cancer. We established C6/lacZ glioma tumors in immunocompetent Sprague-Dawley rats and subsequently administered concentrated vTKiGFP retrovector intra-tumorally. Intra-tumoral delivery of 9 μ l (~108 retroparticles) of concentrated vTKiGFP retrovector stock did not improve survival of animals who did not subsequently receive gancyclovir. These control rats (tumor+, retrovector+, but no GCV) had a mean survival of 38 days (range 20-52 days). Post-mortem examination of whole brain tissue sections, revealed that efficient and stable tumor-specific gene transfer had occurred (Fig. 7). Transgene expression persisted in the growing tumor as long as rats survived after retrovector administration. Examination of surrounding normal brain tissue failed to reveal GFP fluorescence (Fig. 7) suggesting that retrovector integration and expression occurred in tumor cells only and not in mitotically quiescent neurons, as would be expected from a retroviral vector.

Twelve test rats received GCV following tumor-targeted vTKiGFP delivery. Of these, two died shortly (within two weeks) following the end of GCV treatment. This "acute" death rate attributable to direct GCV toxicity (~16%), is comparable to that observed by other investigators who administered GCV at equal or lesser doses. The mechanism of death is likely related to cytopenia and immunosuppression associated with severe, albeit reversible, bone

marrow toxicity. Surviving test rats fully recovered from GCV toxicity approximately two weeks following its completion.

5 All of the test rats remained alive and well more than 80 days post tumor implantation. Two rats developed symptomatic tumor recurrences and were sacrificed on day 82 post tumor implantation (Fig. 8). Examination of brain
10 tissue sections on these late relapses, revealed large tumors with areas of green fluorescence inter-spaced with GFP-negative tumor cells (Fig. 7). This suggests that recurrence was due in part to growth of untransduced tumor cells, or of tumor cells in which the retrovector was silenced following integration. The presence of
15 GFP+ tumor cells suggests that the GCV regimen was not intensive and/or durable enough to eliminate all transduced tumor cells in these rats. Alternatively, a subset of transduced, TK-expressing cells may have acquired resistance to gancyclovir via some other means. Lastly, the "bystander" effect - especially its immune effector arm - may vary in intensity from animal to animal.
20 This may explain the observed pattern of late relapses, suggesting that that there was a early "suicide/bystander" effect which led to increased survival but that some tumor cells - transduced or not - "escaped" from the bystander effect and eventually led to a recurrence. However, the sum of the suicide and bystander effect was clearly sufficient to enhance survival of a majority of animals (66%) who received vTKiGFP and GCV. Our observed long-term survival rate (>120 days) is substantially greater than that observed following intra-tumoral injection of TK retroviral producer cells and compares favorably with that obtained with suicide adenovectors, including those incorporating tumor-specific promoter elements.

25 In the experimental group, 2/12 animals died from GCV toxicity and 2/12 succumbed to late tumor recurrences. These data suggest that GCV dose reduction would be desirable to lessen toxicity, however the duration of treatment may need to be extended to allow elimination of all gene modified cells. The relatively late recurrences (day 82 post implant), lead us to speculate that the
30 "immune" bystander effect may have been mitigated in these two animals. It may be possible to increase the immune response by co-administering immunomodu-

latory genes (IL2, GMCSF) with TK such as has been described by others. Furthermore, it may be useful to re-administer the suicide retrovector to those animals who have residual disease following a cycle of therapy, and to repeat this until maximal response has been achieved. However, it is unknown if a specific -
5 and neutralizing - immune response against VSVG-typed retroparticles will be elicited.

This constitutes the first report of *in vivo* delivery of a cell-free retrovector concentrate with tumor-specific, high efficiency gene transfer and expression, with evident biologically significant anti-tumor activity. We propose
10 that concentrated vTKiGFP retrovector may be of therapeutic value for humans with brain cancer. The high-titer of the concentrated reagent would allow intra-tumor delivery of a useful retrovector dose without the risks of injecting relatively large volumes in a confined space (such as brain). vTKiGFP targeting of a tumor mass *in vivo* should subsequently lead to its regression, and the bystander effect
15 may have a significant impact on the biology of local and distant micrometastatic glioma deposits within the neuropil. This and related therapeutic reagents may also be useful in the treatment of other locally advanced and metastatic malignancies.

The present invention will be more readily understood by referring to
20 the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Retrovector encoding for GFP & HSVTK fusion protein serves as a versatile 25 suicide/reporter instrument for cell and gene therapy applications

In accordance with a preferred embodiment of the present invention, a pseudotyped retrovector encoding for a chimeric GFP/HSVTK fusion protein that serves as a bifunctional suicide transgene and reporter was designed. The fusion
30 gene was incorporated in a VSV-G pseudotyped retrovector (vGFP/TKfus) and high titer stable retroviral producer generated ($\sim 3 \times 10^6$ retroparticles/ml). Rodent tumor cell lines transduced at an MOI of 15 for three days led to 100% gene

transfer efficiency and southern blot analysis confirmed that unrearranged proviral genomes integrated in chromosomal DNA. Protein extract immunoblot with HSVTK anti-sera revealed the presence of a 70kd protein consistent with the predicted size of a HSVTK+GFP fusion protein. Cell growth of cell lines
5 expressing vGFP/TKfus was significantly suppressed in the presence of gancyclovir thereby confirming functionality of the HSVTK C-terminal component of the fusion protein. Fluorescence microscopy and FACS analysis revealed that GFPTKfus-mediated fluorescence was 30 fold greater than that observed in an equivalent bicistronic HSVTK&GFP vector. Interestingly, the
10 fusion protein was consistently and preferentially localized in the nucleus. Normal human peripheral blood T-lymphocytes were phytohemagglutinin-activated and expanded in IL2-containing media. Co-culture with vGFPTKfus producer cells for three days led to 100% gene transfer and expression with nucleus-restricted green fluorescence as observed in transduced tumor cell lines.
15 The DA3 mouse mammary carcinoma cell line was transduced with vGFP/TKfus and implanted in syngenic Balb/c mice. Pre-established tumors completely regressed in 7/9 mice treated with gancyclovir. In conclusion, we demonstrate the utility of vGFPTKfus as a reporter/suicide transgene in tumor cells *in vitro* and *in vivo*. Furthermore, its potential use as an analytical and therapeutic tool targeting
20 human T-lymphocytes in adoptive cell therapy applications are shown.

Materials and Methods

Cell lines and plasmids

The pMC1TK plasmid containing the HSVTK cDNA was graciously provided by Gerald Batist (Lady Davis Institute for Medical Research, Montreal,
25 QC). The pJ6Σbleo plasmid and 293GPG retroviral packaging cell line were generous gifts from Richard. C. Mulligan (Children's Hospital, Boston, MA). MSCV-Neo plasmid was kindly provided by Robert G. Hawley (The Toronto Hospital, Toronto, ON). The pGFPC1 plasmid was purchased from Clontech. A bicistronic retroviral expression vector encoding for HSVTK and GFP (pTKiGFP)
30 has been previously described by us. The DA3 mouse mammary adenocarcinoma cell line and the A549 human lung carcinoma cell line were generously provided

by Moulai Alaoui-Jamali (Lady Davis Institute for Medical Research, Montreal, QC).

Retroviral vectors

The synthesis of the GFPTK fusion retrovector was as follows. A
5 1177 base pair fragment containing the entire HSVTK cDNA was excised from
the plasmid pMC1TK by a *HincII-XmaI* digest and ligated with 4693 bp fragment
generated from a *Ecl136II-XmaI* digest of pEGFP-C1 (Clontech). This fused
HSVTK sequence at the 3' end of GFP sequence whilst maintaining coding
sequences in frame (Fig. 9, panel A). The fused gene product was then imported
10 into our previously described retroviral expression vector. This product was
labeled pGFP/TKfus. Transduction of target cells with pGFP/Tkfus -derived
retroviral particles (vGFP/TKfus) will lead to the stable incorporation of LTR
flanked proviral genome. pGFP was generated by replacing the Neo coding
sequence from MSCV-Neo plasmid with the cDNA of GFP (Fig. 9, panel B).

Retroviral gene transfer

DA3 mouse mammary cells were plated at 2×10^5 cells per well in a 24
well dish and allowed to adhere. Media was removed and replaced with 500 μ L
of thawed, retrovirus conditioned media collected from stably transfected
293GPG retroviral producers was added (MOI of ~ 8). Polybrene (Sigma) was
20 added to a final concentration of 6 μ g/ml. This procedure was repeated daily for
three consecutive days. Stably transduced DA3 cells were subsequently
expanded. No clonal selection was performed, and mixed populations of
transduced cells were used for all subsequent experiments. As shown in Fig. 10,
Southern blot analysis was performed on 15 μ g of overnight *NheI* digested
25 genomic DNA extracted from stably transduced cells as well as untransduced
control cells. Blots were hybridized with a P^{32} labeled, full-length 700 bp GFP
cDNA probe, washed and exposed on photographic film.

Growth suppression assay and Western blot analysis

Stably transduced test and control cells were trypsinized and plated at
30 a density of 1000 cells per well in a flat bottomed tissue-culture treated 96 well
plate (Costar corporation, Cambridge, MA). Clinical-grade gancyclovir (GCV,

Hoffman-Laroche, Mississauga, ONT) was added to achieve a range of concentrations from 0.01 to 5000 µg/ml in a final volume of 100 µL of RPMI/10%FBS. Cells were incubated at 37°C and media was replaced with fresh GCV after three days for a total exposure of 6 days. The percentage of surviving cells was measured using a method based on the metabolism, by living cells, of the mitochondrial substrate 3-(4,5-deimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) into formazan, which is detected by measurement of the optical density at 570nm(40). Percent Survival is calculated as follows $[\text{OD}_{570} \text{ test} - \text{OD}_{570} \text{ empty well}] / [\text{OD}_{570} \text{ untreated cells} - \text{OD}_{570} \text{ empty well}] \times 100$. All data points were measured in triplicate in at least three separate experiments.

For the detection of the HSVTK protein in transduced cells, 20ug of total protein from either untransduced DA3 cells or DA3 cells transduced with vGFP/Tkfus or vTKiGFP was separated by SDS-PAGE transferred to nitrocellulose (BioRad). Detection of HSVTK-containing proteins was done using polyclonal rabbit anti-HSVTK (Yale University) and detection by an Enhanced Chemiluminescence Detection kit (Amersham). Blots were exposed to Kodak X-OMAT film for 5 min.

Flow cytometry and fluorescence microscopy

Flow cytometric analysis was performed within two weeks following transduction to ascertain retrovector expression and gene transfer efficiency as measured by GFP fluorescence. In brief, adherent transduced cells were trypsinized and resuspended in RPMI at $\sim 10^5$ cells per ml. Analysis was performed on a Epics XL/MCL Coulter analyzer. Live cells were gated based on FSC/SSC profile and analyzed for GFP fluorescence. Fluorescence microscopy was performed as follows. Transduced cells were plated over 22mm square microscope cover glasses previously placed in wells of 6-well flat bottom tissue culture plates. Once cells reached subconfluency, they were washed with phosphate buffered saline (PBS) three times, fixed by exposing to 3% paraformaldehyde for 15mins at room temperature, and washed again several times with PBS. The cover glasses were then removed and mounted on pre-

cleaned frosted end microscope slides (Fisher Scientific) using gelvatol. Photographs of cells under fluorescence microscopy (excitation of 470 nm) were taken utilizing a Olympus BX60 microscope attached to a Compaq Deskpro computer. Pro-Series Capture 128 Image-Pro Plus Software was used with an
5 integration time of 10 seconds.

Animal model of cancer

Female Balb/c mice were implanted subcutaneously with 1×10^6 DA3 mouse mammary cells transduced invitro with vGFP/TKfus or vGFP. Five days post-implant the mice were given 150mg/kg of GCV twice a day i.p. for five
10 consecutive days. From day 10 post-implant, tumour volume at implantation site was assessed by palpation. Discernible tumors were measured every three days by caliper measurements.

Results

GFP and HSVTK fusion protein design and synthesis

15 A fusion cDNA was synthesized where the entire HSVTK coding sequence, including 71 nucleotides from the 5' untranslated (5'UTR) HSVTK sequence were fused to the truncated 3' end of GFP cDNA (see Materials and methods for details). The resulting linker region between GFP and HSVTK coding sequence and predicted translation product are shown in Fig. 9, panel A.
20 The resulting fusion protein is constituted of GFP to which is fused a 24 AA peptide linker derived from translation of the HSVTK 5'UTR. The endogenous HSVTK start codon and subsequent entire coding sequence extending past the STOP codon leads to a full-length HSVTK protein as the C-terminal half of this chimera. The coding sequence for the eGFP and HSVTK fusion protein
25 (GFPTKfus) was incorporated in a plasmid retroviral expression vector we have previously described (REF) to generate pGFPTKfus (Fig. 9, panel B). Retrovector plasmids expressing GFP only (pGFP) and a bicistronic vector encoding for HSVTK and GFP (pTKiGFP) are also depicted in Fig. 9, panel B. These plasmid vector constructs were utilized to generate retroviral producer cell
30 lines derived from the 293GPG retroviral packaging cell line as previously described. Following co-transfection of 293GPG cells with pGFPTKfus and

BLEO, and Zeocin drug selection, a mixed population of cells (293GFPTKfus) was maintained. The 293GFPTKfus retroviral producer cells will generate Vesicular Stomatitis Virus-G protein (VSVG) pseudotyped retroparticles following tetracycline withdrawal. The retroviral titer generated from the vGFPTKfus producer was 3×10^6 particles/ml as measured on the human A549 lung carcinoma cell line. We performed DNA analysis of vGFPTKfus transduced A549 cells to determine if the replication-defective pro-viral genome integrated in chromosomal DNA unrearranged. NheI digest of genomic DNA from vGFPTKfus-transduced cells will generate a 3.6kb DNA fragment encompassing LTR-flanked vector sequences. Southern blot of NheI digested DNA was hybridized with a GFP sequence-specific probe and a DNA fragment of predicted size is detected (Arrow, Fig. 10).

Green fluorescence in retrovirally transduced cells

Green fluorescence (~530 nm) emitted from cells excited with "blue" (470 nm bandwidth) light serves as a reporter of GFP transgene expression in genetically-engineered cells. We sought to compare green fluorescence emission in DA3 mouse mammary carcinoma cells transduced with VSVG-pseudotyped retrovectors encoding GFP alone (vGFP), HSVTK and GFP as part of a bicistronic retrovector (vTKiGFP) or the GFPTKfus protein (vGFPTKfus). All cells were transduced at a MOI of ~8 for three consecutive days to generate three polyclonal populations of cells. No clonal selection was performed and green fluorescence was measured by flow cytometry. As shown in Fig. 11, the mean fluorescence intensity (MFI) of DA3/GFPTKfus cells is 100 fold greater than control and 30 fold greater than DA3/TKiGFP cells. The MFI of cells transduced with GFP reporter only (DA3/GFP cells) is ~20 fold greater than that of DA3/GFPTKfus. Fluorescent microscopic examination of DA3/GFPTKfus cells revealed that green fluorescence was dominantly and sharply localized in nucleus, excluded from nucleoli, and that faint cytoplasmic fluorescence was observed in "high expressors" only. In contrast, DA3/GFP cells green fluorescence is distributed evenly between cytoplasm and nucleus (Fig. 12).

HSVTK expression and sensitization to gancyclovir

The expression of immunoreactive HSVTK moiety was directly compared between DA3/GFPTKfus and DA3/TKiGFP cells. Western analysis of whole cell lysates immunoblotted with anti-HSVTK antibody was performed. As shown in Fig. 13, DA3/TKiGFP express the expected 50 kd native HSVTK protein, whilst DA3/GFPTKfus cells bear a 75kd immunoreactive protein whose size is consistent with the predicted mass of GFP (25kd) + HSVTK (50 kd) fusion product. Though of different molecular weight, equivalent amounts of anti-HSVTK immunoreactive protein is generated by both vGFPTKfus and vTKiGFP transduced cells.

We determined if the HSVTK component of GFPTKfus remained functional. Cells genetically-engineered to express the HSVTK suicide gene will convert the prodrug gancyclovir to its cytotoxic phosphorylated metabolite. Sensitization to gancyclovir of retrovirally transduced cells was measured in a growth suppression assay as described in materials and methods. When compared to DA3/GFP cells, both DA3/TKiGFP and DA3GFPTKfus cells are significantly sensitized to gancyclovir with an IC₅₀ ~1000 fold (Fig. 14). Doubling time (~24 hours) for all cell lines are similar.

In Vivo sensitization of transduced tumor cells to gancyclovir

DA3 cells are tumorigenic in Balb/c mice. We determined if pre-established DA3/GFPTKfus subcutaneous tumor implants would regress following gancyclovir treatment *in vivo*. Five days after subcutaneous implantation of DA3 cells, palpable tumors arise. Mice were treated with gancyclovir 150mg/kg intraperitoneally twice daily for five days and presence of palpable tumor assessed over time. 7/9 rodents implanted with DA3GFPTKfus tumors were rendered tumor-free by gancyclovir treatment. Mice in which DA3GFPTKfus tumors regressed completely subsequent to GCV treatment remained tumor-free for at least two months. Tumor volume growth rate over time was identical between GCV-treated DA3GFP implants and mock-treated DA3GFPTKfus implants.

Discussion

The Herpes Simplex Virus TK gene product is a potent conditional suicide gene. Conditional, since its overexpression is innocuous to engineered cells, yet renders these same cells extremely sensitive to the cytotoxic effects of the nucleobase analog gancyclovir. The HSVTK transgene has been a widely adopted tool in the field of gene therapy for adoptive cell therapy and for cancer gene therapy applications.

In its use as a conditional "self-destruct" mechanism, its role is to eliminate engineered cells if their *in vivo* behaviour is undesirable. The best example of this approach is in the treatment of graft versus host disease (GVHD) that may arise following donor lymphocyte infusion (DLI). DLI are commonly administered to patients who suffer a relapse of a hematological malignancy following allogeneic bone marrow transplantation. In the pioneering work of Bonini et al, a bicistronic vector containing expressing the truncated Nerve Growth Factor Receptor reporter (NGFR) and a HSVTK/Neomycin phosphotransferase II fusion gene was retrovirally introduced in donor lymphocytes, selected in G418, and subsequently administered to patients. *In vivo* tracking of engineered lymphocytes was achieved by tracking cell surface expression of NGFR which can be readily detected by flow cytometry and immunohistochemistry. Patients in whom GVHD arose as a complication of DLI were treated with GCV and offending engineered lymphocytes were eliminated. In some patients GVHD reversed. For this and related strategies, pre-infusion dominant selection of HSVTK expressing lymphocytes is mandatory. Further, post-infusion tracking of cells in blood, lymphoid organs and diseased tissue serves well the purpose of deciphering the role of gene-marked donor lymphocytes in GVHD and the effect of GCV treatment on its control. HSVTK/GCV tandem serves a potent and effective "self-destruct" switch, however the requirement of dominant selection and traceable marker, make for bulky multigenic vector constructs, limiting the ability to introduce other useful therapeutic transgenes within retroviral constructs of limited (~8-10kb) gene packing space. Genetic engineering of autologous lymphocytes and other

immunocompetent cells are also attracting a significant amount of attention as tools for therapy of acquired diseases, such as AIDS and cancer. Many of these cell therapy reagents will also require a dominant selectable marker and a conditional "self-destruct" switch, especially if an unforeseen undesirable phenotype arises from their use in clinical studies.

Similar requirements are also relevant to the use of HSVTK as an anti-cancer "killer" gene. HSVTK gene transfer to pre-established cancer will lead to tumor regression. Effectiveness is wholly dependent on gene transfer efficiency *in vivo*, and issues related to *in vivo* transgene tracking are critical to ascertain cause/effect relationship. Consequently, a dominant selectable marker/reporter/suicide multivalent transgene would greatly enhance the utility of a "self-destruct" or "suicide" switch in gene and cell therapy applications.

Fluorescent reporter proteins such as the Green Fluorescent Protein (GFP) have properties well suited as a combined selectable marker/reporter. First, their intra-cellular expression can be readily detected in live cells without need of fixation, antibodies or affinity columns, allowing rapid and specific cell sorting - based on green fluorescence - with standard flow cytometry equipment as described by others. Secondly, *in vivo* tracking of live engineered cells is facilitated. We have also shown that tumor-targeted GFP reporter gene transfer can also be directly visualized by fluorescence microscopy. Combining HSVTK and fluorescent protein expression would address the objective of combining reporter/marker with a potent "self-destruct" gene. We have addressed this previously with the use of a HSVTK and GFP reporter bicistronic vector construct. However, we have found that to obtain optimal retrovirus-driven HSVTK protein production, that the GFP cDNA had to be incorporated in the less favorable IRES-dependent translation position. Though GFP reporter expression is readily detectable in some tumor and tissue type, its expression level often falls below satisfactory levels, especially when utilizing fluorescent microscopy. Further, engineering of multigenic retroviral constructs incorporating HSVTK and GFP with other synergistic anti-cancer immunomodulatory genes, such as IL2,

GMCSF and others, is limited by the inadequacies of multicistronic constructs where multiple IRES will inevitably yield poor transgene protein production.

We examined the utility of a GFP & HSVTK fusion protein as a bifunctional reporter/marker and suicide transgene as part of a therapeutic retrovector. As depicted in Fig. 9, we generated a fusion gene where HSVTK is fused to the C-terminus of GFP with a 24 amino acid linker. This construct was incorporated in a retroviral expression vector and VSVG-pseudotyped retroparticles were generated as a gene transfer vehicle. We show that the GFPTKfus coding sequence was permissive for high-titer virus production and that retroviral constructs bearing this sequence were genetically stable upon integration in target cells as shown by southern blot analysis (Fig. 10). Target cells transduced at an equivalent MOI reveal that GFPTKfus expression leads to a degree of green fluorescence that is markedly superior to that seen with either negative control (100 fold) or a bicistronic TKiresGFP construct (30 fold), yet is less "bright" than a monocistronic GFP expression vector (Fig. 11). Interestingly, we observed that the GFPTKfus protein localized predominantly to the nucleus of transduced cells as opposed to the diffuse pancellular distribution of native GFP protein (Fig. 12). This data strongly suggests that HSVTK protein contains a nuclear localizing signal that may play a role in the normal physiological role of this viral protein. Immunoblot analysis of whole cell extracts with anti-HSVTK antibody revealed that mass of GFPTKfus protein was 75kd, consistent with the predicted combined mass of GFP (25kd) and HSVTK (50kd) (Fig. 13). Further, it is noted that equivalent amounts of TK-immunoreactive protein was generated from the GFPTKfus and TKiGFP retroviral constructs, consistent with the prediction that the HSVTK protein translation levels obtained from the 5' cistron of a bicistronic construct (TKiGFP) are equivalent to a related monocistronic construct (GFPTKfus). We found that acquired GCV sensitivity was identical between cells transduced with either vTKiGFP or vGFPTKfus retrovectors (Fig. 14). These biochemical observations were confirmed in a mouse tumor implant model where pre-established GFPTKfus-expressing tumors could be eradicated in a majority of mice by GCV treatment.

The sum of these data suggest that the GFP fusion protein bearing a C-terminus fused HSVTK loses some of its intrinsic "brightness" when compared with GFP only. Yet, the fusion GFPTKfus protein conserves the ability to sensitize cells to GCV as well as native HSVTK. Though native GFP protein is
5 "brightest", its expression levels - derived from a bicistronic construct designed to optimize HSVTK expression (TKiGFP, Fig. 9) - are substantially lower than that obtained from the GFPTKfus retroviral construct. Therefore, the GFPTKfus gene product wholly preserves its vital "self-destruct" and "suicide" feature and exhibits desirable fluorescent properties superior to that achieved in fluorescent
10 bicistronic constructs designed to optimize HSVTK expression. In conclusion, GFPTKfus can serve as an improved substitute to bicistronic HSVTK and GFP constructs, where "suicide" characteristics are preserved and green fluorescent reporter expression levels are superior. Further, incorporation of GFPTKfus in multigenic vector constructs where other gene products of interest are included
15 will greatly facilitate their characterisation in cell and gene therapy applications, including dominant selection by cell sorting, analysis of vector expression in live cells *in vitro* and *in vivo*, and biologically relevant expression of a potent "self-destruct" and "suicide" transgene.

While the invention has been described in connection with specific
20 embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be
25 applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A retroviral particle for delivering a gene to a tumor tissue cell, said retroviral particle being pseudotyped with a vesicular stomatitis virus G (VSV G) protein.
2. A tumor-specific retroviral expression vector comprising a suitable promoter, a retroviral untranslated sequence including a packaging sequence and a primer building site, a cloning site preferably linked to an internal ribosomal entry site (IRES), said IRES being operably linked to a first nucleotide sequence encoding a suitable marker, and a retroviral 3' long terminal repeat (LTR) sequence, for expressing a second nucleotide sequence inserted in said cloning site.
3. A retroviral expression vector according to claim 2, wherein said second nucleotide sequence comprises a therapeutic gene.
4. A retroviral expression vector according to claim 3, wherein said therapeutic gene comprises a suicide gene.
5. A retroviral expression vector according to claim 4, wherein said suicide gene is TK.
6. A retroviral expression vector according to claim 4, wherein said second nucleotide sequence encodes a Herpes simplex virus thymidine kinase.
7. A retroviral expression vector according to claim 5 or 6, wherein said marker comprises a green fluorescent protein (GFP).
8. A retroviral expression vector according to claim 5 or 7, wherein said a first and second nucleotide sequences are combined to encode a GFP/TK fusion protein.
9. A plasmid encoding a bicistronic, non-splicing murine retrovector comprising a multiple cloning site (MCS) operably linked to an enhanced green

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fluorescent (EGFP) reporter (AP2) for transferring a provirus to a target cell and expressing said provirus into said target cell, for co-expressing a nucleotide sequence inserted into said plasmid with said EGFP reporter within a bicistronic framework.

10. A replication-defective retroviral expression vector comprising a suitable promoter, a retroviral untranslated sequence including a packaging sequence and a primer building site, a multiple cloning site (MCS) operably linked to an internal ribosomal entry site (IRES), said IRES being operably linked to a first nucleotide sequence encoding a suitable marker and, a retroviral 3' long terminal repeat (LTR) sequence, for expressing a second DNA sequence inserted in said MCS.

11. An expression vector according to claim 10, wherein said marker comprises an enhanced green fluorescent protein (EGFP).

12. An expression vector according to claim 10, wherein said promoter comprises a CMV promoter.

13. A method for treating a tumor, the method comprising administering to a mammal suspected of having a tumor a retroviral expression vector comprising a first nucleotide sequence, said first nucleotide sequence being therapeutic, and a second nucleotide sequence encoding a marker, said first and second nucleotide sequences being co-dominantly expressed, and administering to said mammal a nucleobase analog.

14. A method for detecting *in vivo* a genetically modified cell with an expression vector according to claim 9 to a tumor tissue cell of a mammal, the method comprising administering a retroviral expression vector comprising a first nucleotide sequence encoding a retrovirus and a second nucleotide sequence encoding a marker, said first and second nucleotide sequences being co-dominantly expressed, and detecting the expression of said second nucleotide sequence by using one of fluorescence microscopy and flow cytometry techniques.

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15. A method for producing a retroviral particle according to claim 1, the method comprising stably transfecting a suitable cell line with the expression vector of claim 9.

16. A method for producing retroviral particles, the method comprising transfecting a suitable cell line with the expression vector of claim 10 and transfecting said cell line with a drug resistance plasmid.

17. The cell line obtained by the method according to claim 14.

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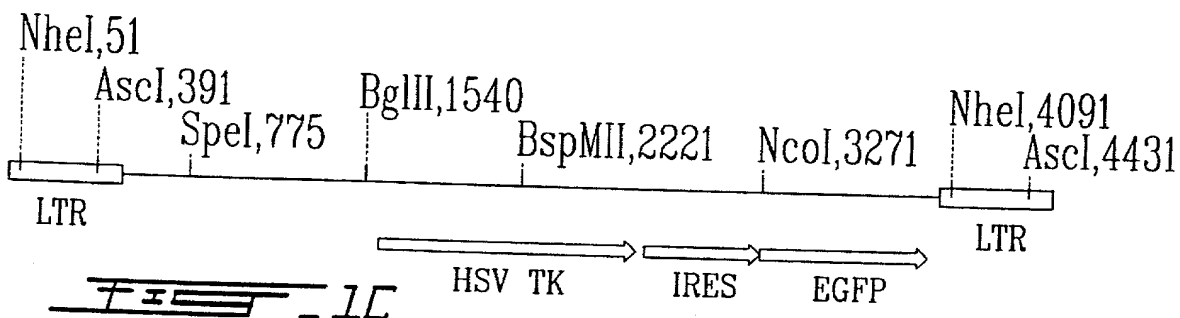
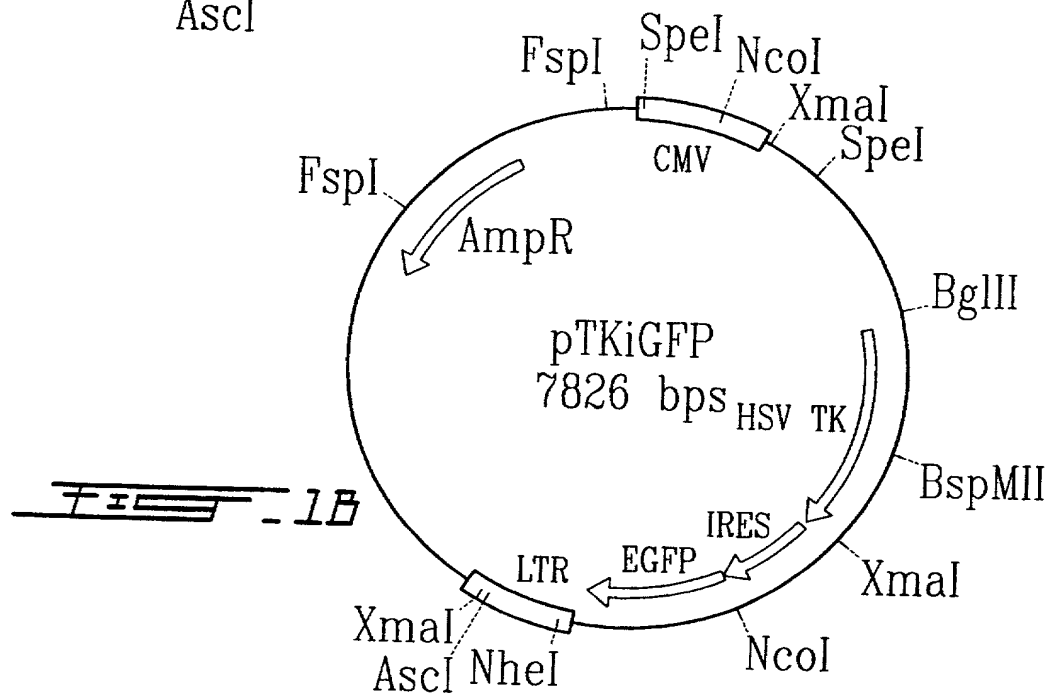
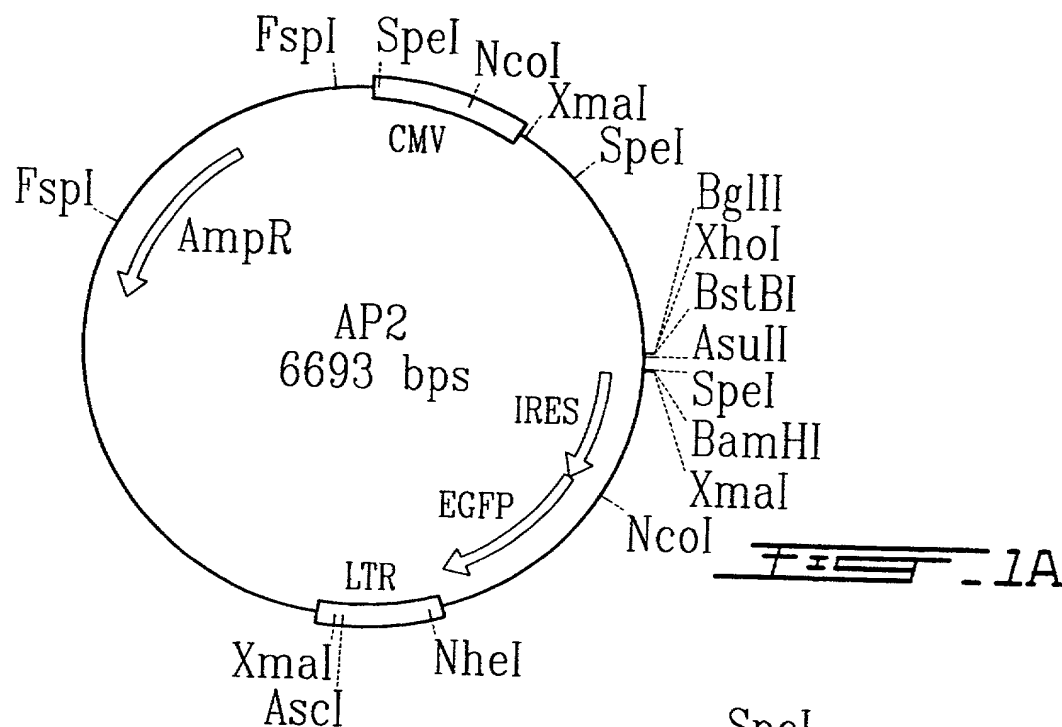
(54) Title: PSEUDOTYPED RETROVIRAL VECTOR FOR GENE THERAPY OF CANCER

(57) Abstract: The invention relates to retroviral expression vectors and more particularly to pseudotyped retroviral vectors for gene therapy of cancer. Direct *in vivo* tumor-targeting with "suicide" viral vectors is limited by inefficient gene transfer and indiscriminate transfer of a conditionally toxic gene to surrounding non-malignant tissue. Retrovectors pseudotyped with a Vesicular Stomatitis Virus G protein (VSVG) may serve as a remedy to this conundrum. These retroviral particles differ from standard murine retroviruses by their very broad tropism and the capacity to be concentrated by ultracentrifugation without loss of activity. A VSVG-typed retrovector can be utilized for efficient and tumor specific Herpes Simplex Virus Thymidine Kinase (TK) gene delivery *in vivo*. A bicistronic retroviral vector which expresses TK and Green Fluorescence Protein (pTKiGFP) was constructed.

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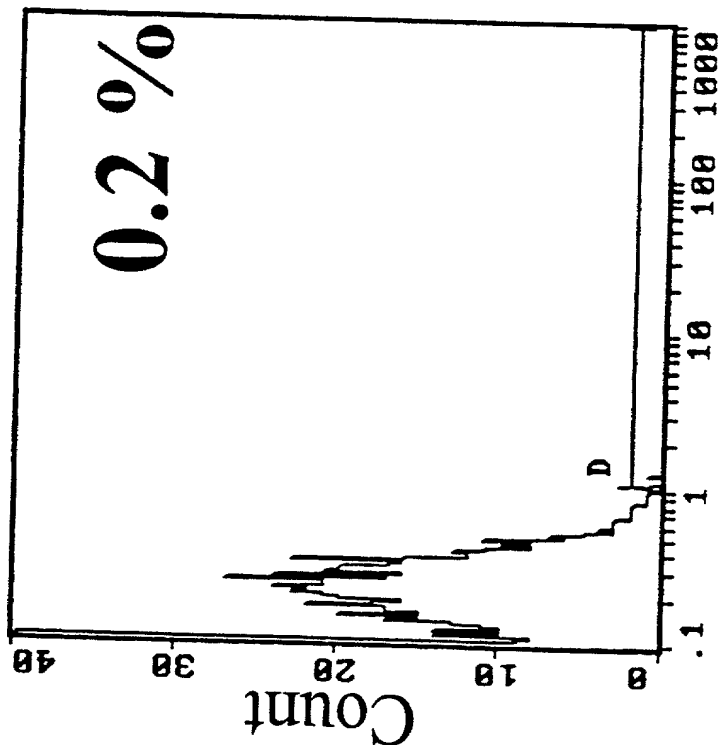
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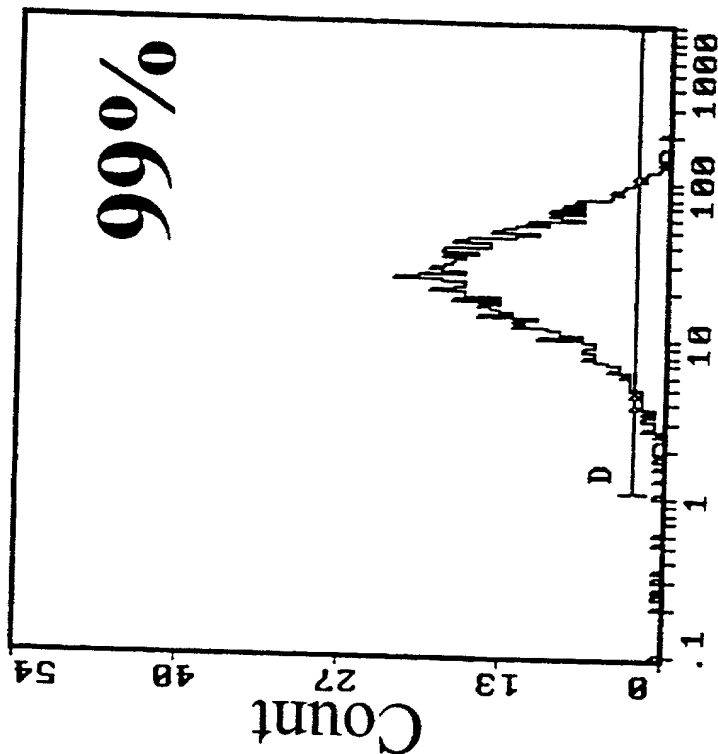
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UWR7 human glioma cells

Null



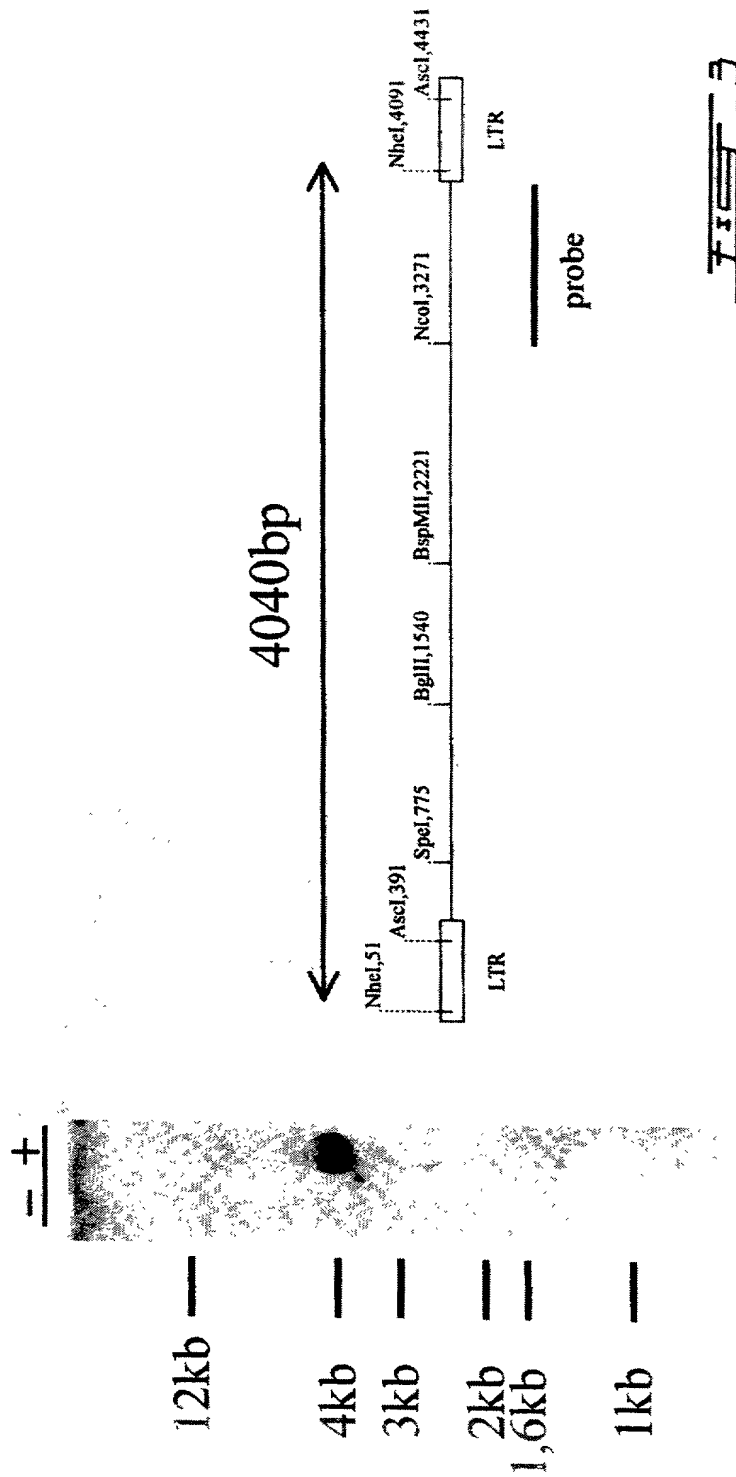
Transduced



Green fluorescence 

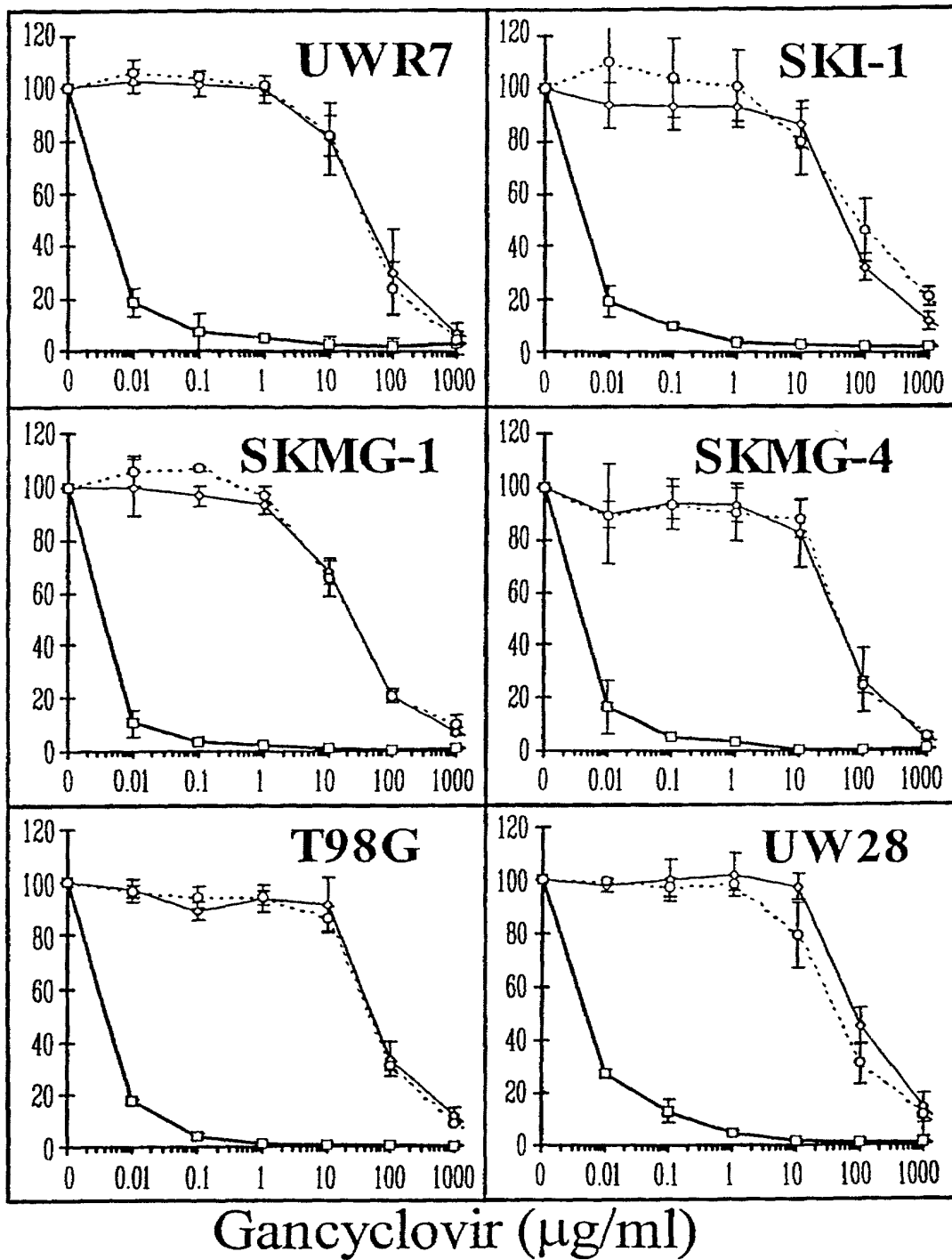


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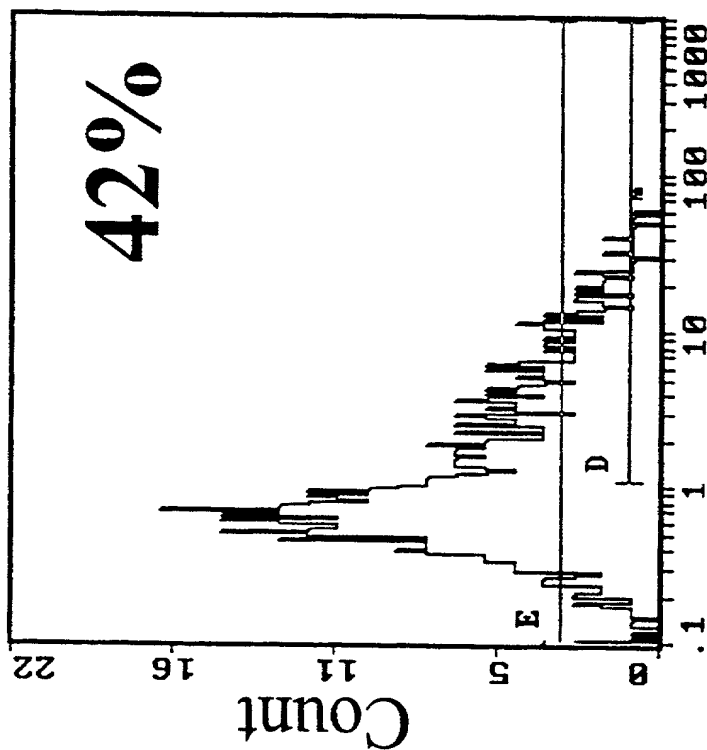
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Percent Survival

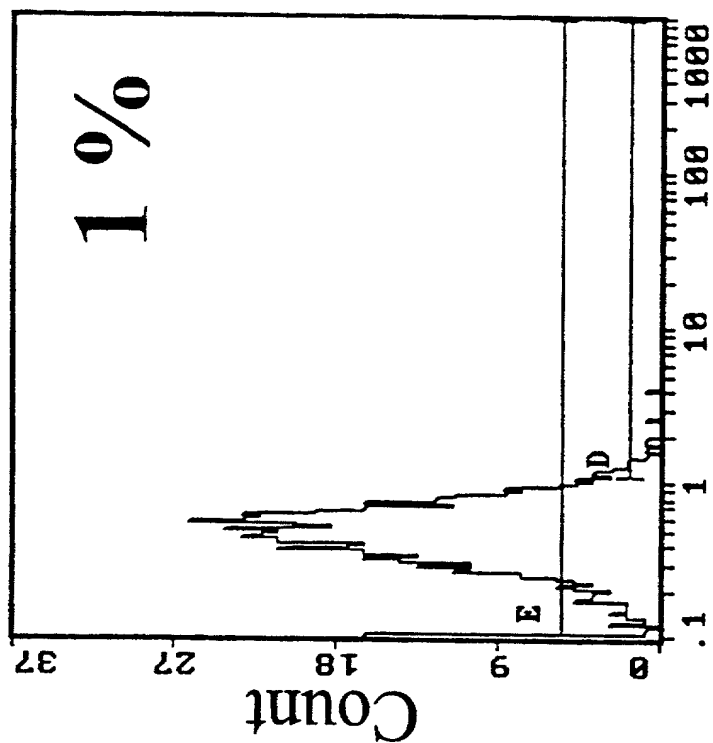
FIG - 4

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293AP3



293GPG null




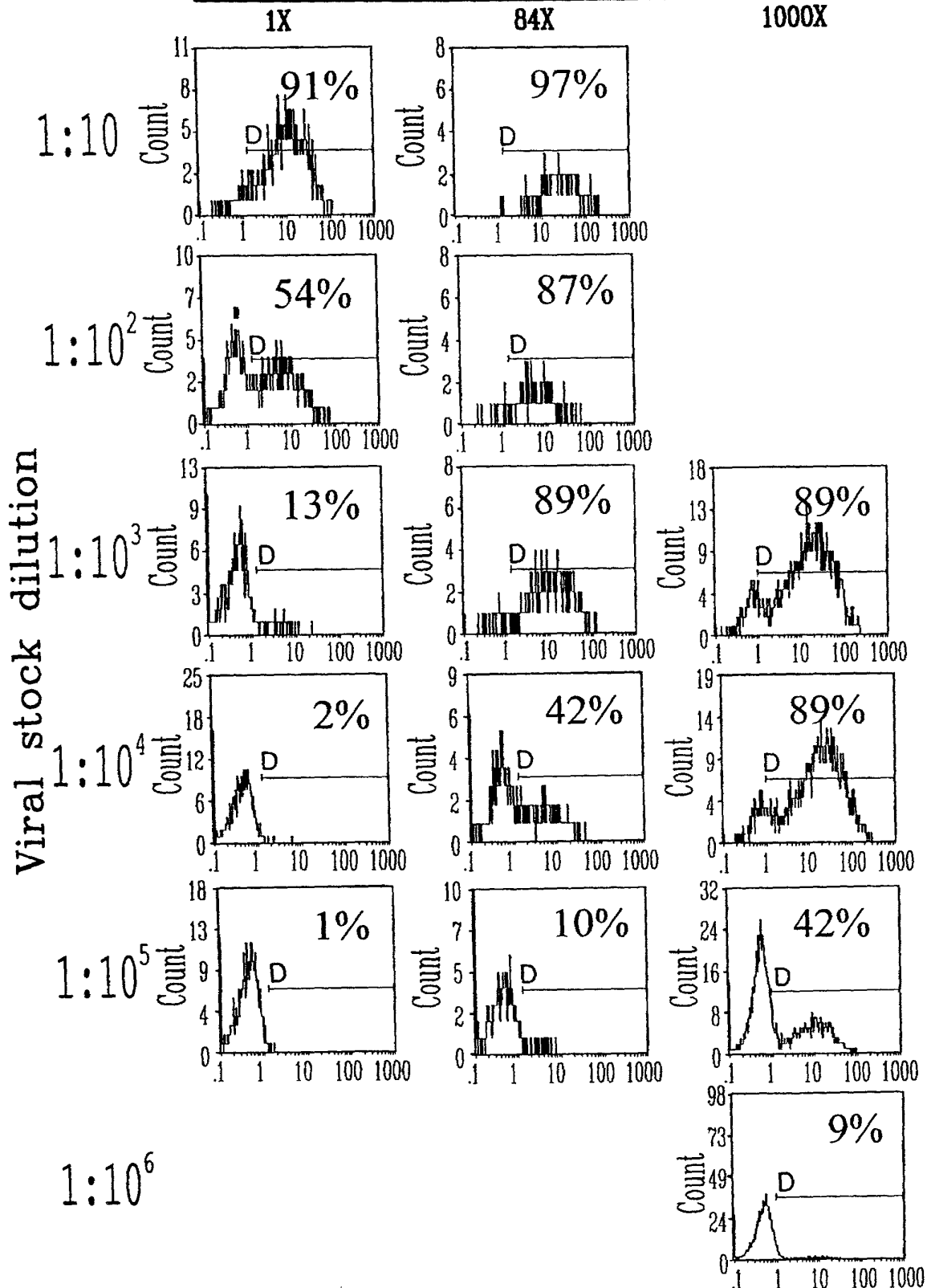
Green fluorescence 

Fig 5

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Viral stock concentration



Green fluorescence →

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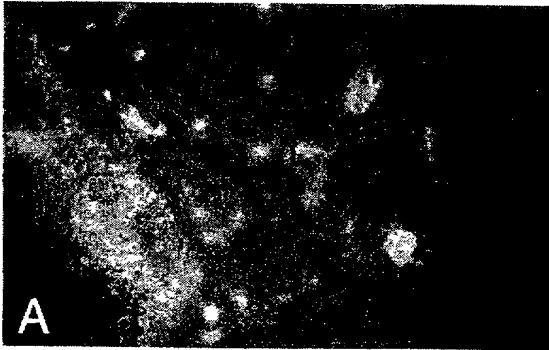


FIG. 7A

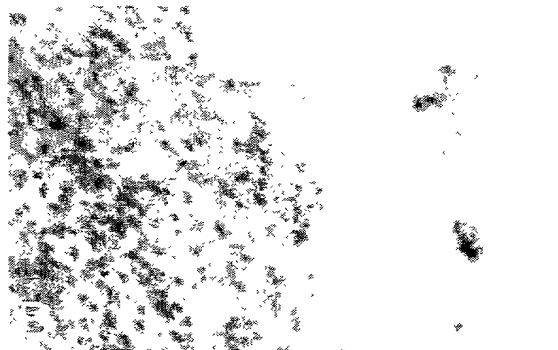


FIG. 7B

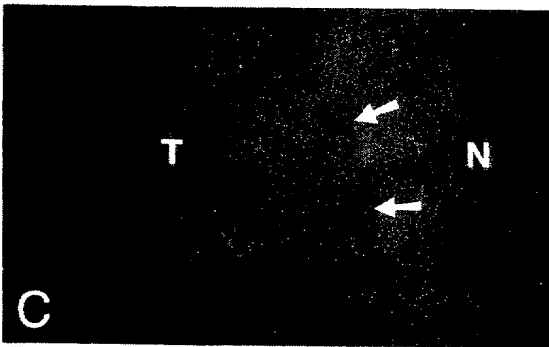


FIG. 7C

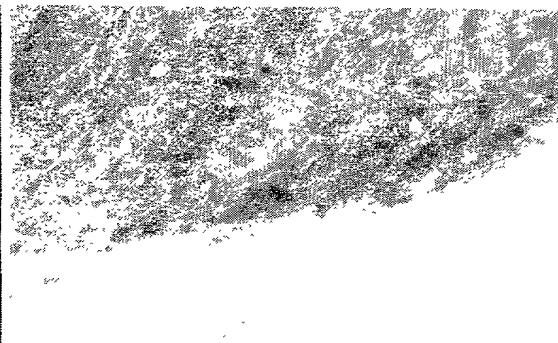


FIG. 7D

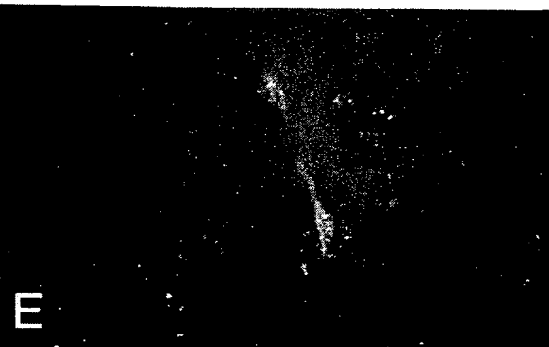
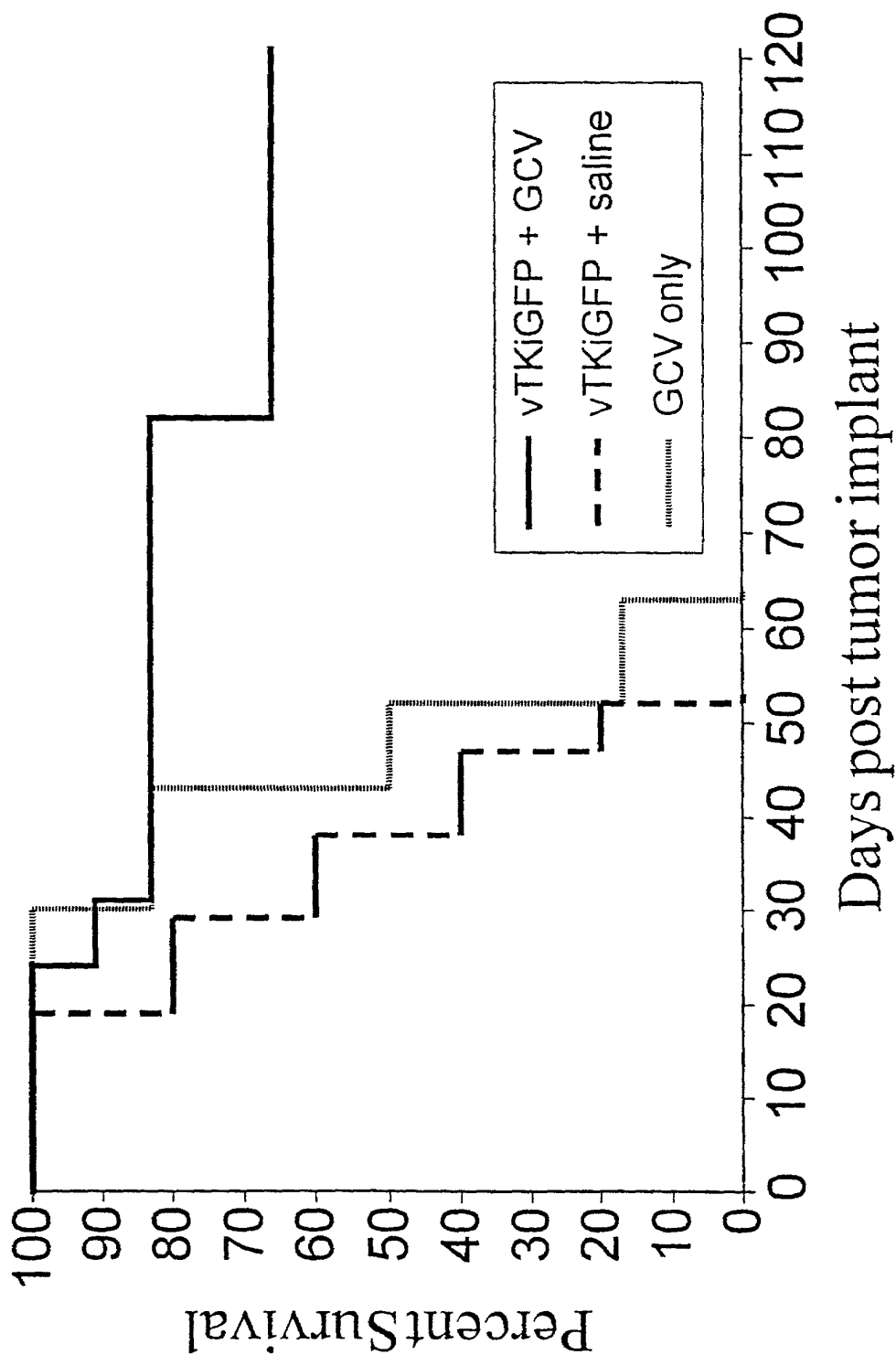


FIG. 7E



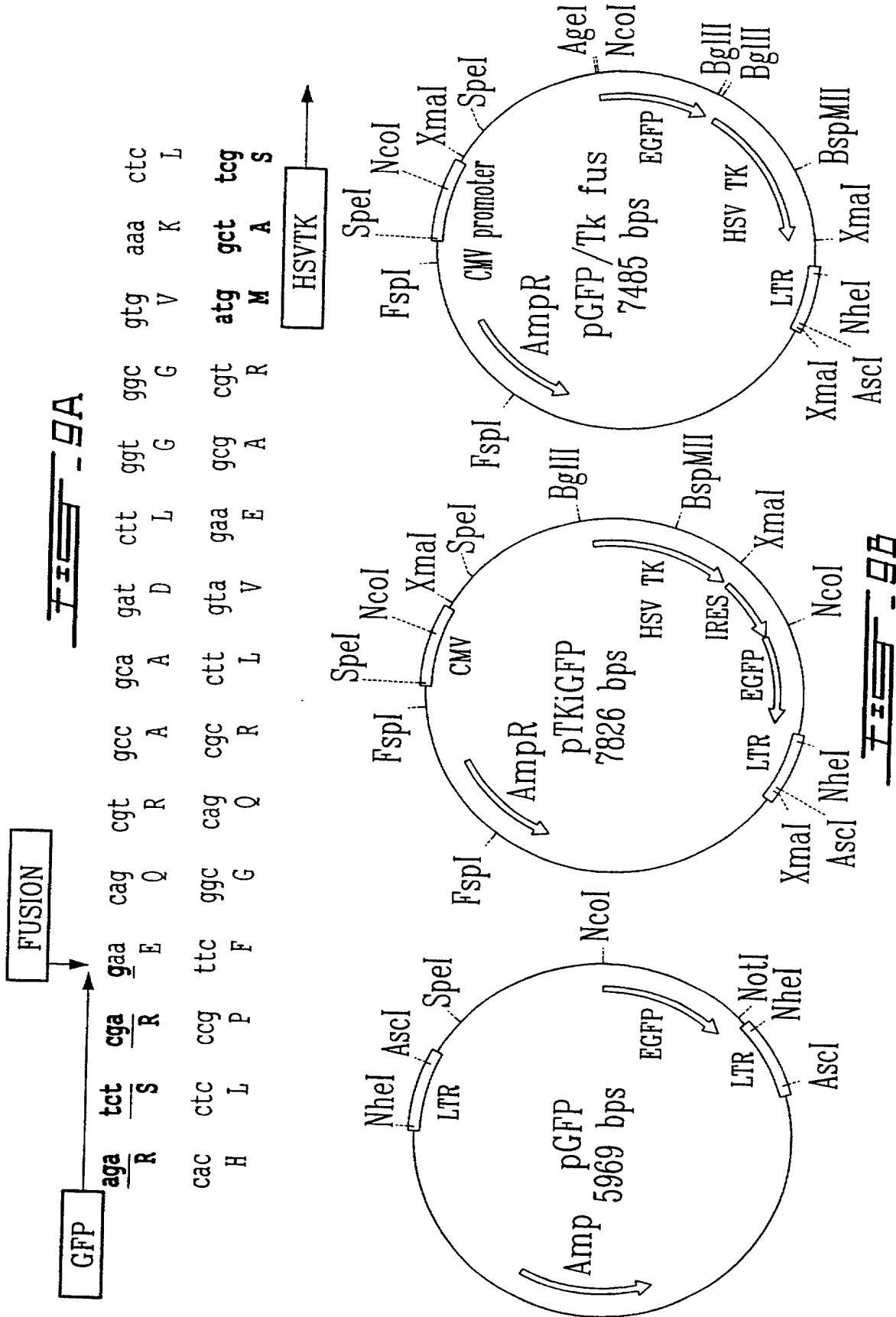
FIG. 7F

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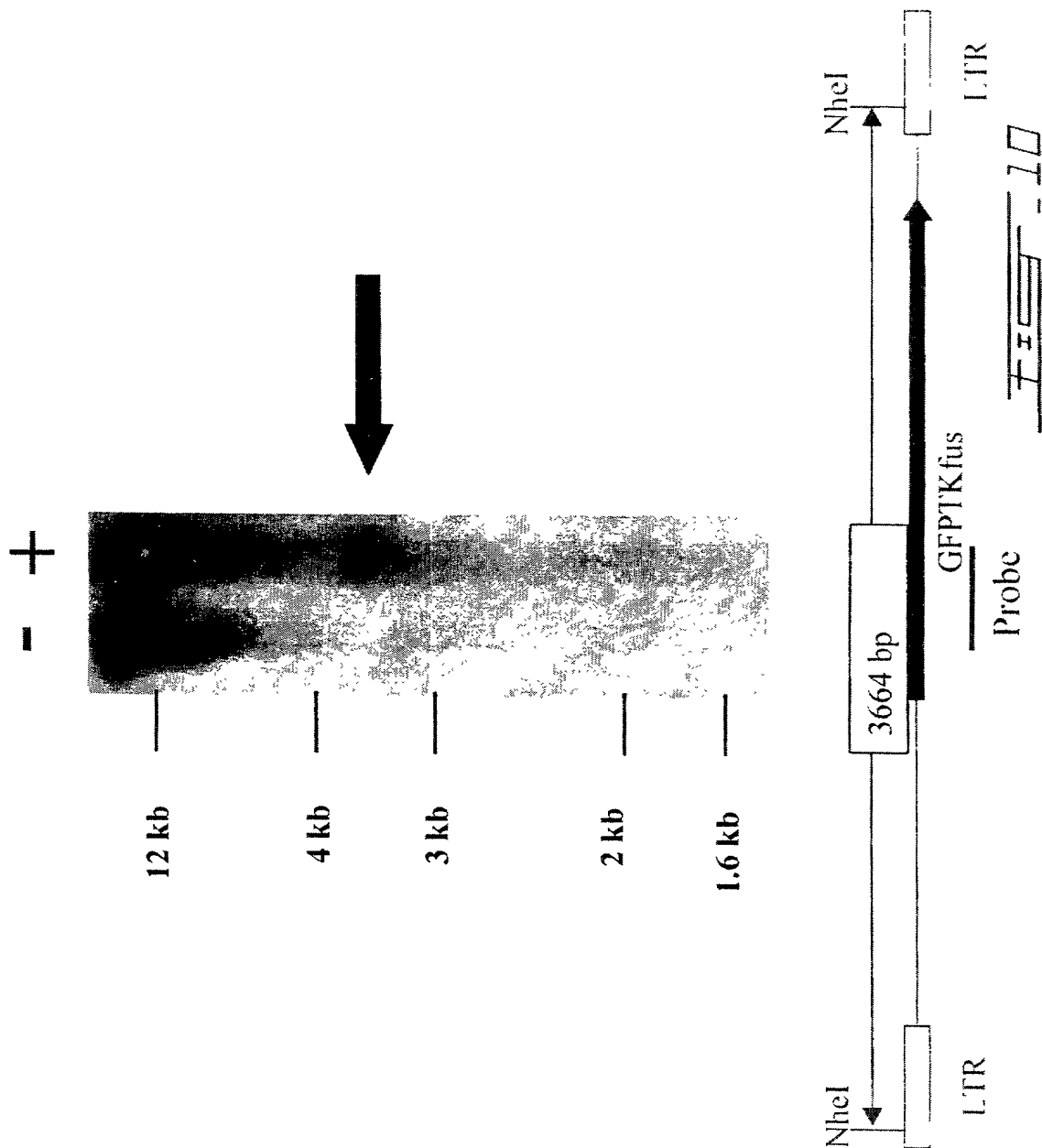


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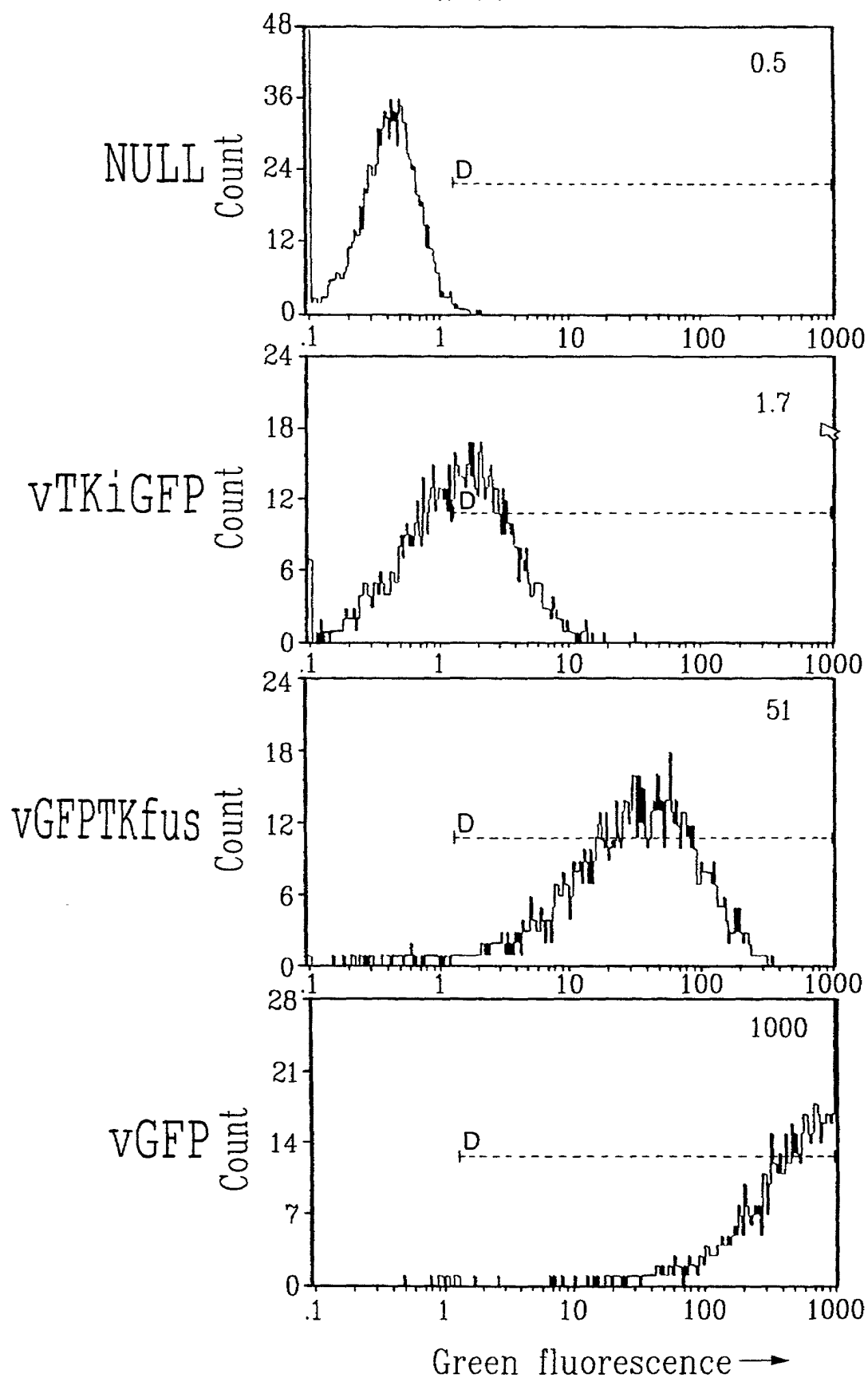
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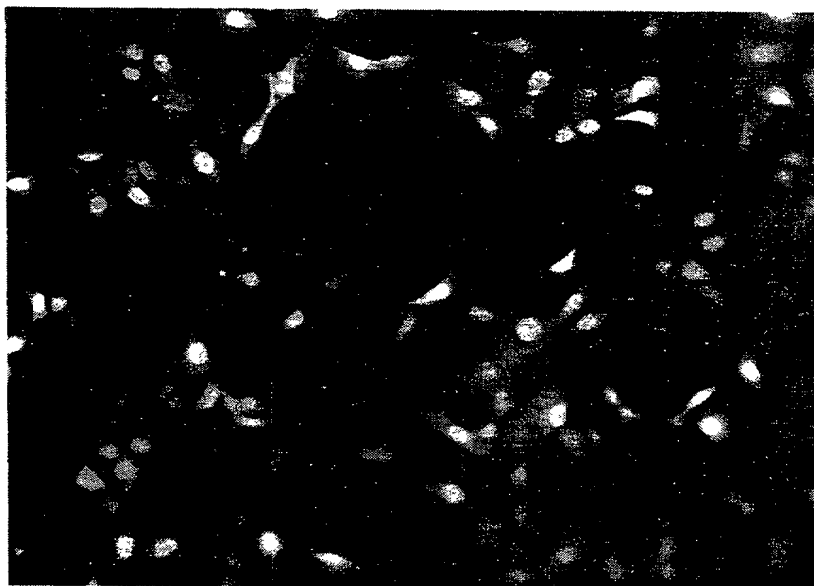


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DA3GFPTKfus



DA3GFP

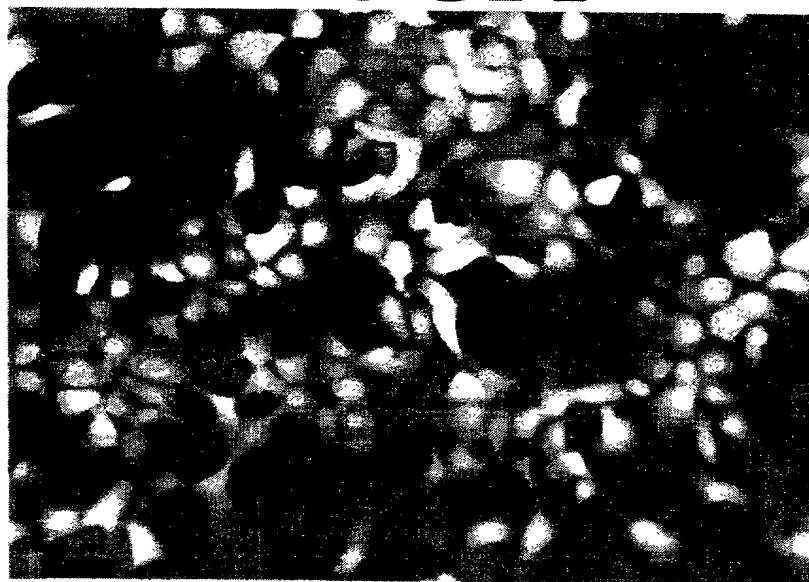
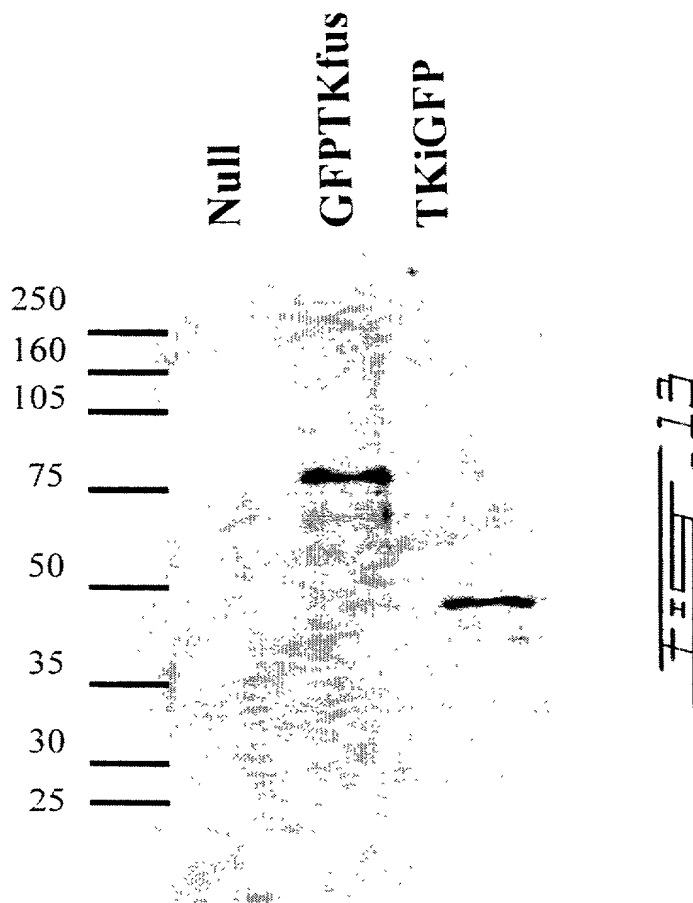
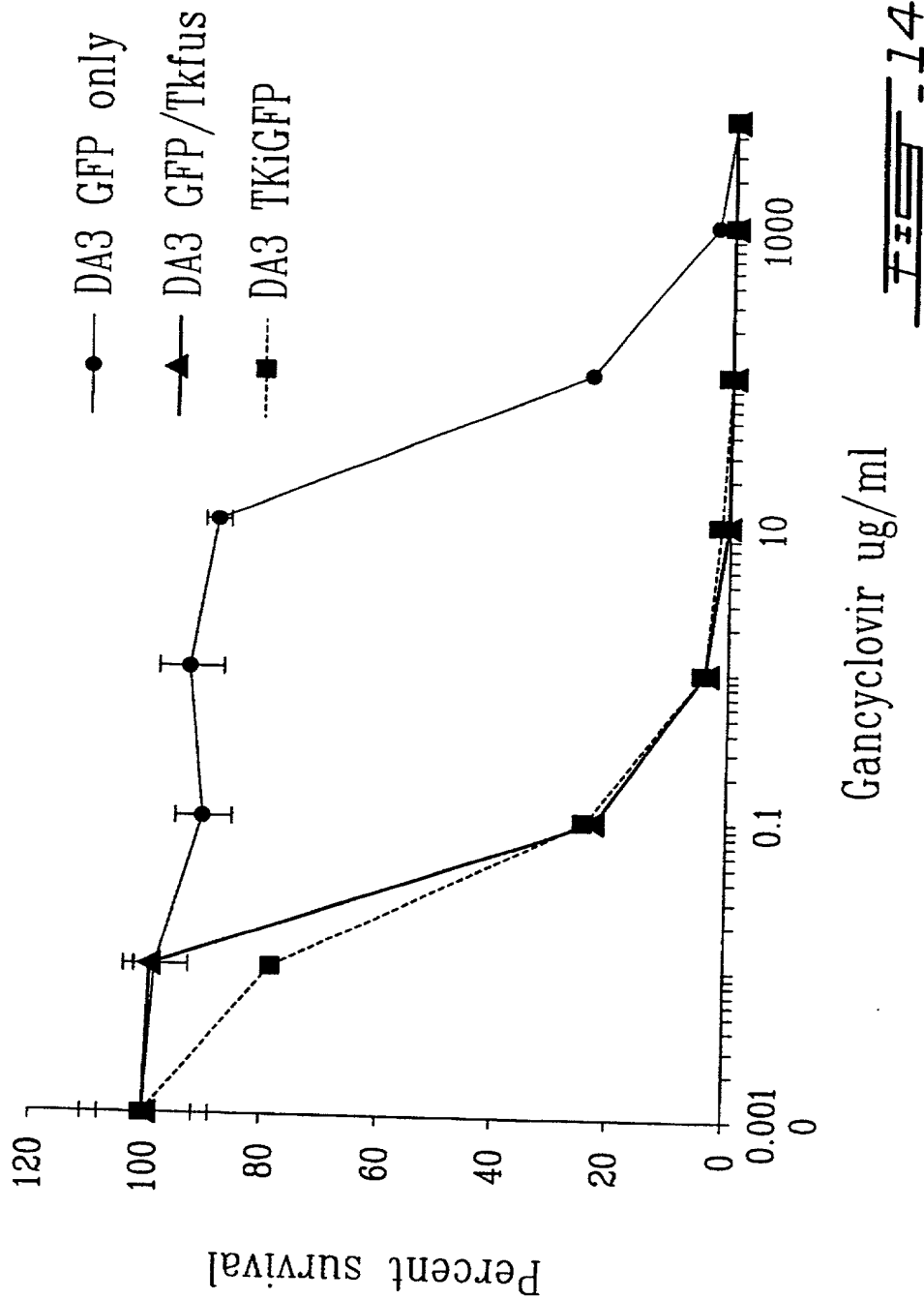


FIG. 12

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DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below under my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**PSEUDOTYPED RETROVIRAL VECTOR FOR GENE
THERAPY OF CANCER**

the Specification of which

☐ is attached hereto

☒ was filed on April 20, 2000

as International Application No. PCT/CA00/00445

I hereby state that I have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

<u>APPLICATION</u> <u>NUMBER</u>	<u>PRIOR FOREIGN FILED APPLICATION(S)</u> <u>COUNTRY</u> <u>(MONTH/DAY/YYYY)</u>	<u>PRIORITY</u> <u>CLAIMED</u>
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I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER(S)
60/130,680

FILING DATE (MM/DD/YYYY)
April 23, 1999

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this

Attorney Docket No.: 2626-1-001

application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent <u>Application No.</u>	PCT Parent <u>Number</u>	Parent Filing <u>(MM/DD/YYYY)</u>	Parent Patent <u>Number (if applicable)</u>
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Attorney Docket No.: 2626-1-001

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